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PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re application of: Peter Lind et al.

Confirmation No: 7317

Serial No: 09/750,373

Group Art Unit: 1647

Filed: December 28, 2000

Examiner: Robert S. Landsman

For: POLYNUCLEOTIDES ENCODING G-PROTEIN COUPLED RECEPTORS AND USES THEREOF

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APPELLANT'S BRIEF PURSUANT TO 37 C.F.R. § 1.192

Appellants hereby submit an original and two copies of this Appeal Brief to the Board of Patent Appeals and Interferences ("the Board") in response to the Final Office Action mailed on August 18, 2003. The Notice of Appeal was timely submitted on February 17, 2004 and was received in the Patent and Trademark Office ("the Office") on February 17, 2004. This Appeal Brief is timely submitted in light of the concurrently filed Petition for an Extension of Time of five months to and including September 17, 2004. Authorization to deduct the fee as required under 37 C.F.R. §1.17(a)(2) from Appellants' Representatives' deposit account. The Commissioner is also authorized to charge the fee for filing this Appeal Brief ($330.00), as required under 37 C.F.R. §1.17(c), to Cozen O'Connor Deposit Account No. 50-1275.

Appellants believe no fees in addition to the fee for filing the Appeal Brief and the fee for the extension of time are due in connection with this Appeal Brief. However, should any additional fees under 37 C.F.R. §§1.16 to 1.21 be required for any reason related to this communication, the Commissioner is authorized to charge any underpayment or credit any overpayment to Cozen O'Connor Deposit Account No. 50-1275.

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I. REAL PARTY IN INTEREST

The real party in interest in the above-identified patent application is Pharmacia and Upjohn Company. An Assignment to Pharmacia and Upjohn Company was recorded at Reel 012077, Frame 0511 on August 21, 2001.

II. RELATED APPEALS AND INTERFERENCES

Applicants undersigned attorney is unaware of any related appeal or interference that will affect or be affected by or have any bearing on the decision rendered in this appeal.

III. STATUS OF CLAIMS

The present application was filed with claims 1-89. Claims 2-6, 11, 26-28 were cancelled during prosecution. Claims 1, 9, 10, 16, 24, 25, and 29-31 were amended during prosecution. Claims 1, 7-10, 12-25, and 29-33 are on appeal and appear in Appendix A. Claims 34-89 were withdrawn from consideration pursuant to 37 C.F.R. § 1.142(b).

Claims 1, 7-10, 12-25, and 29-33 stand or fall together.

IV. STATUS OF AMENDMENTS

All amendments have been entered.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The present invention relates to Appellants' discovery and identification of novel human polynucleotide sequences that encode proteins sharing significant sequence identity with known receptors. Claim 1 of the present application recites "An isolated nucleic acid molecule comprising a nucleotide sequence that encodes a polypeptide comprising an amino acid sequence of SEQ ID NO: 25." The specification teaches that SEQ ID NO:25 represents the amino acid sequence of nGPCR-1007. (Table 1, page 13 of specification).

The claimed receptors share significant sequence identity with a gene known to be involved in asthma and diabetes. The specification as filed recites that the claimed receptor is "useful in the treatment of respiratory ailments such as asthma, where T cells are implicated by

the disease." (see, specification, page 62). The specification teaches that the claimed receptor is useful in the treatment and diagnosis of diabetes (see, *inter alia,* specification, page 99). The specification also teaches that the claimed receptor shares homology with the conopressin 2 receptor, an arginine vasopressin receptor. (see, specification, page 74). Arginine vasopressin receptors are known to be involved in asthma as well as in diabetes. Accordingly, the present invention is useful, *inter alia*, in the diagnosis and treatment of asthma and diabetes.

In addition, the claimed receptors have a number of other uses, including the localization of the gene or encoded protein *in vivo* or *in vitro*. Gene expression patterns as well as tissue expression profiles can be attained using the present invention. Methods of generating antibodies specific for the claimed receptors are also set forth in the specification. Appellants point out that a specific working example of tissue expression profiling of the nGPCR-1007 gene product is set forth in the application as filed as Example 5. Examples of chromosomal localization are also described in the application as filed at Example. Another use of the receptors involves mapping the sequences to a specific region of a human chromosome.

VI. GROUNDS OF REJECTIONS TO BE REVIEWED ON APPEAL

The issues in this appeal are whether claims 1, 7-10, 12-25, and 29-33 comply with the requirements of 35 U.S.C. §§ 101 and 112, first paragraph (enablement).

VII. ARGUMENT

In the Advisory Action dated December 24, 2003, the Office maintained the final rejection of claims 1, 7-10, 12-25, and 29-33 under 35 U.S.C. §§ 101 and 112, first paragraph (enablement).

A. Claims 1, 7-10, 12-25, and 29-33 comply with the requirements of 35 U.S.C. §101

The rejection of Claims 1, 7-10, 12-25, and 29-33 under 35 U.S.C. § 101 is improper and should be reversed because the claimed invention is supported by a specific, substantial and credible asserted utility as well as a well-established utility.

In the Office Action mailed August 18, 2003, the Office maintained the rejection under U.S.C. §101. The Office indicated that no specific or substantial utility had been provided for the claimed receptor, noting that the "specification does not disclose any function, nor any dysfunction, associated with altered levels or forms of the polynucleotide or polypeptide of the claimed invention." (Office Action mailed August 18, 2003, pages 5-6).

The supporting written description of the present application discloses several specific and substantial utilities that are credible and well-established. The utilities asserted in the specification as originally filed have been confirmed. Specifically, Appellants point out that:

1) BLAST searches show significant similarity between the claimed receptors and known receptors involved in asthma. Highest scoring matches show between about 82% and 84% percent similarity to two isoforms of GPRA receptors (also known as "G protein-coupled receptor for asthma susceptibility" or "GPR154") (BLAST search results, pages 5-6, attached hereto as Appendix B; NCBI Sequence Viewer for NP_997055, attached hereto as Appendix C; NCBI Sequence Viewer for NP_997056, attached hereto as Appendix D);

2) GPRA receptors are known to play a role in asthma and are particularly important in asthma susceptibility. (Laitinen *et al.*, 2004, Science, 304:300-304; copy attached as Appendix E);

3) BLAST searches show significant similarity between the claimed receptors and known arginine vasopressin receptors. Highest scoring matches show between about 82% and 84% percent similarity to a vasopressin receptor (known as "VRR1") (BLAST search results, pages 5-6, attached hereto as Appendix B; NCBI Sequence Viewer for NP_997055, attached hereto as Appendix C; NCBI Sequence Viewer for NP_997056, attached hereto as Appendix D);

4) the conopressin 2 receptor is an arginine vasopressin receptor and is said to be involved in the "pathogenesis of asthma and other IgE-mediated diseases." (copy of Entrez Gene report attached hereto as Appendix F; Shimuara *et al.*, Acta Paediatr. Jpn. 1990, 32(2) 197-200; attached hereto as Appendix G); and

5) arginine vasopressin receptors are known to be involved in diabetes (Thibonnier, Current Hypertension Reports 2004, 6:21-26, Current Science, Inc. ISSN 1522-6417 (hereinafter "Thibonnier I"), copy attached hereto as Appendix H).

Accordingly, the supporting written description of the present application discloses utilities that are specific, substantial and credible. The asserted utilities are also well-established and have been confirmed in the literature.

35 U.S.C. §101

To satisfy 35 U.S.C. §101, an invention must be useful. 35 U.S.C. §101 states:

> Whoever invents or discovers any ***new and useful*** process, machine, manufacture, or composition of matter, or any new and useful improvement thereof may obtain a patent therefore, subject to the conditions and requirements of this title. (emphasis added).

The MPEP states that "[t]o violate [35 U.S.C. §] 101 the claimed device must be totally incapable of achieving a useful result." MPEP 2107, citing *Brooktree Corp. v. Advanced Micro Devices,* Inc., 977 F.2d 1555, 24 USPQ2d 1401, 1412 (Fed. Cir. 1992) (underlining in original). Additionally, this section of the MPEP cites *E.I. du Pont De Nemours and Co. v. Berkeley and Co.* (620 F.2d 1247, 1260 n.17 (8th Cir. 1980) for the proposition that "the defense of non-utility cannot be sustained without proof of total incapacity." Appellants note that "utility" under 35 U.S.C. §101 is assessed from a "minimal" perspective. Almost *any* evidence of utility (excluding "throw away" utilities) is sufficient under 35 U.S.C. §101.

A recent decision from the Court of Appeals for the Federal Circuit is instructive in this regard. In *Juicy Whip Inc. v. Orange Bang Inc.*, 51 USPQ2d 1700 (Fed. Cir. 1999), the claimed invention was directed to a "post-mix" beverage dispenser. In an action for infringement, summary judgment was granted in favor of the alleged infringer, based on the conclusion that the claimed invention lacked utility and was therefore unpatentable under 35 U.S.C. §101. The Federal Circuit reversed, relying on *Manson* for support. As noted by the Federal Circuit:

> The threshold of utility ***is not high***: An invention is useful under Section 101 if it is capable of providing ***some identifiable benefit.***

(51 USPQ2d at 1702; emphasis added).

Utility Examination Guidelines

The Utility Examination Guidelines were promulgated to assist Office personnel in their review of applications for compliance with the utility requirement under 35 U.S.C. §101. The Guidelines require that a claimed invention have a specific, substantial and credible asserted utility, or, alternatively a well-established utility that is immediately apparent, or implied by the specification's disclosure of the properties of a material, alone or taken with the knowledge of one skilled in the art.

Section 2107 of the MPEP sets forth "Guidelines for Examination of Applications for Compliance with the Utility Requirement". The Examination Guidelines instruct the Examiner to:

> (A) ***Read the claims and the supporting written description.***
> (1) Determine what the applicant has claimed, noting any ***specific embodiments of the invention.***
> (2) Ensure that the claims define statutory subject matter (i.e., a process, machine, manufacture, composition of matter, or improvement thereof).
> (3) If at any time during the examination, it becomes readily apparent that the claimed invention has a well-established utility, do not impose a rejection based on lack of utility. An invention has a well established utility if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (e.g., properties or applications of a product or process), and (ii) the utility is specific, substantial, and credible.
>
> (B) ***Review the claims and the supporting written description to determine if the applicant has asserted for the claimed invention any specific and substantial utility that is credible***:
> (1) If the applicant has asserted that the claimed invention is useful for any particular practical purpose (i.e., it has a "specific and substantial utility") and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility.
> (i) A claimed invention must have a specific and substantial utility. This requirement excludes "throw-away," "insubstantial," or "nonspecific" utilities, such as the use of a complex invention as landfill, as a way of satisfying the utility requirement of 35 U.S.C. 101.

> (ii) Credibility is assessed from the perspective of one of ordinary skill in the art in view of the disclosure and any other evidence of record (e.g., test data, affidavits or declarations from experts in the art, patents or printed publications) that is probative of the applicant's assertions. An applicant need only provide one credible assertion of specific and substantial utility for each claimed invention to satisfy the utility requirement.

(Examination Guidelines; emphasis added).

The Office failed to "Read the claims and the supporting written description"

As set forth in Appellants' Response to Office Action dated July 24, 2003, and Appellants' Response to Final Rejection dated November 17, 2003, the claimed invention is supported by a specific, substantial and credible utility. Specific and substantial utilities that are credible are set forth in the application as filed. Appellants respectfully assert that the Office has failed to follow the guidelines in its review of the present application for compliance with the utility requirement.

For example, as set forth in the Office Action mailed February 6, 2003, the Examiner alleges that "no comparison to any known GPCR could be found in the specification." The Examiner also alleges that "the specification fails to teach the skilled artisan the utility of the claimed polynucleotide of SEQ ID NO: 12 and the protein of SEQ ID NO:25, which are only believed to be GPCRs." (February 6, 2003 Office Action, page 4).

It appears clear that the Examiner failed to read the supporting written description of the present application. The specification identifies credible specific and substantial utilities for the claimed invention. As discussed above, the specification does indeed provide a comparison to a known GPCR, setting forth that the claimed receptor has homology to the conopressin receptor 2. The specification does teach "the utility of the claimed polynucleotide of SEQ ID NO: 12 and the protein of SEQ ID NO:25", stating that the claimed receptor is useful in the treatment of asthma and diabetes.

Each of the utilities asserted in the supporting written description of the present application is specific, substantial and credible. Further, each utility is well-established. The asserted utilities do not represent "throw-away," "insubstantial," or "nonspecific" utilities.

Accordingly, those of skill in the art would readily agree that the claimed receptor was useful based in the characteristics of the invention set forth in the supporting written description and therefore the pending claims comply with the requirements of 35 U.S.C. §101.

Specific Utility

The claimed invention has at least one specific utility. MPEP § 2107.01 states that a specific utility is "*specific* to the subject matter claimed." (italics in original).

The utilities asserted in the supporting written description of the present specification are specific to the subject matter claimed. As discussed above, the claimed receptor exhibits about 84% sequence identity to GPRA receptors. GPRA receptors are known to play a role in asthma and are particularly important in asthma susceptibility. The claimed receptor exhibits about 84% sequence identity to arginine vasopressin receptors which are involved in the "pathogenesis of asthma and other IgE-mediated diseases" as well as diabetes. Accordingly, the claimed receptors are specific for the diagnosis and treatment of asthma, diabetes and other IgE-mediated diseases.

Substantial Utility

The claimed invention has a substantial utility. Section 2107.01 of the MPEP states that "any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a 'substantial' utility." The use of the claimed invention in diagnosis and treatment of asthma, diabetes and other IgE-mediated diseases provides a definite public benefit and, accordingly, provides a 'substantial' utility.

Credible Utility

The utilities asserted by Appellants is credible. The level of sequence identity between the claimed receptor and the known, asthma-related receptor and the arginine vasopressin receptor is about 84%. This identity alone would lead "a person of ordinary skill in the art to

conclude that the asserted utility is more likely than not true." (see MPEP § 2107.02; underlining in original).

Further the Guidelines comment on the use of computer based analysis of nucleic acids to assign functions to a nucleic acid or polypeptide based upon homology to sequences found in databases. Specifically, the Guidelines state that the:

> When a class of proteins is defined such that the members share a specific, substantial, and credible utility, the reasonable assignment of a new protein to the class of sufficiently conserved proteins would impute the same specific, substantial, and credible utility to the assigned protein. If the preponderance of the evidence of record, or of sound scientific reasoning, casts doubt upon such an asserted utility, the examiner should reject the claim for lack of utility under 35 U.S.C. 101. For example, where a class of proteins is defined by common structural features, but evidence shows that the members of the class do not share a specific, substantial functional attribute or utility, despite having structural features in common, membership in the class may not impute a specific, substantial, and credible utility to a new member of the class. When there is a reason to doubt the functional protein assignment, the utility examination may turn to whether or not the asserted protein encoded by a claimed nucleic acid has a well-established use. If there is a well-established utility for the protein and the claimed nucleic acid, the claim would meet the requirements for utility under 35 U.S.C. 101. If not, the burden shifts to the applicant to provide evidence supporting a well-established utility. There is no per se rule regarding homology, and each application must be judged on its own merits.

(Guidelines). Based on sequence similarity to known receptors, the claimed receptor should be assigned the "same specific, substantial, and credible utility ... ".

The Examiner has also alleged that "even if the specification asserted that the disclosed proteins have biological activities similar to known GPCRS [which it does], this cannot be accepted in the absence of supporting evidence, because generally, the art acknowledges that function cannot be predicted solely on structural similarity to a protein found in the sequence database." (Office Action mailed February 6, 2003, page 4).

However, in addition to sequence identity, the claimed receptors contain additional functional as well as structural motifs characteristic of arginine vasopressin receptors. Such

characteristics are described in Thibonnier I, citing Thibonnier *et al.*, Molecular pharmacology of human vasopressin receptors. *Adv Exp Med Biol* 1998, 449:251-276 (hereinafter "Thibonnier II; copy attached as Appendix I) which recites several features of arginine vasopressin receptors that are present in the claimed receptors. For example, the claimed receptors contain an "aspartate-arginine-tyrosine (Asp[D]- Arg[R]-Tyr[Y]) sequence (DRY motif) in the proximal region of intracellular loop (IL) 2 controlling specificity of G-protein coupling", "[p]roline residues in the TM regions, key to receptor folding and processing", an "asparagine-proline-any amino acid-tyrosine (Asn-Pro-X-X-Tyr) motif in TM7 involved in receptor internalization and signaling", a "cysteine (two adjacent ones in the case of AVP receptors) in the C-terminal domain, which can be palmitoylated, forming a fourth IL", and a cluster of G protein-related kinase (GRK)-phosphorylated serines in the C-terminal domain, which modulates receptor internalization and rate of desensitization". (Thibonnier I, pages 21-22).

Appellants point out that "Courts have routinely found evidence of structural similarity to a compound known to have a particular therapeutic or pharmacological utility as being supportive of an assertion of therapeutic utility for a new compound." (MPEP § 2107.03 citing *In re Jolles*, 628 F.2d 1322, 206 USPQ 885 (CCPA 1980). That the "evidence of structural similarity to a compound known to have a particular therapeutic or pharmacological utility" includes functional motifs provides compelling evidence that the same specific, substantial, and credible utility of the known receptors should be assigned the presently claimed receptors.

Furthermore, Appellants point out that further supporting evidence is not required either under 35 U.S.C. §101 or under the Utility Examination Guidelines. Requiring such supporting evidence would force those attempting to patent inventions in the biotechnological field to provide proofs not required in other fields of endeavor. The legal standard for utility is, and should be, the same in biotechnology as it is in other fields such as organic chemistry because these scientific fields entail the same degree of predictability in extrapolating from one compound or biological molecule to another. For example, just as proteins have secondary and tertiary conformations that may impart some particular properties to the proteins, organic chemicals (*e.g.*, small molecules) have stearic requirements that may impart certain properties to those molecules. In chemical cases pharmaceutical utility is assumed for a number of molecules

with a common core structure and an R group pendant off the core structure (where R as a substituent can be any alkyl, aryl, amino, etc. group), even though utility is only shown, if at all, for one of this family of compounds. In fact, in many instances a mere statement of utility, speculated based upon structural analogy with a known compound which has the stated utility, is accepted as sufficient for organic chemicals. Accordingly, when a utility is known for one protein or polypeptide, utility should be assumed for the family of proteins that share a common biological function or structural characteristic. In the present case, the shared structural characteristic, as discussed above is two-fold. The claimed receptor shares sequence similarity as well as structural elements with proteins known to be useful.

The structural similarities, including ***both*** sequence identity and conserved motifs, between the claimed receptors and the known receptors, therefore, support Appellants' assertion of utility and would lead the art skilled to "conclude that the asserted utility is more likely than not true."

Well-established Utility

MPEP § 2107.02 states that an invention has a well-established utility "if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based in the characteristics of the invention ...". Based on Appellants' assertions of utility, the skilled artisan would readily acknowledge that the invention is useful. The skilled artisan would immediately appreciate that a receptor linked to asthma susceptibility or involved in diabetes was useful.

Summary of §101 Issues

The specification discloses that the claimed receptors are useful in the treatment of asthma and diabetes, utilities that are specific, substantial and credible. The asserted utilities are also well-established. Accordingly, the rejection under 35 U.S.C. §101 cannot stand and should, therefore, be overruled.

B. Claims 1, 7-10, 12-25, and 29-33 are improperly rejected under 35 U.S.C. §112, first paragraph

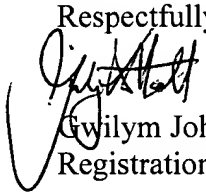
In the Final Rejection mailed August 18, 2003, claims 1, 7-10, 12-25, and 29-33 were again rejected under 35 U.S.C. §112, first paragraph, since allegedly one skilled in the art would not know how to use the invention as the claimed invention is allegedly not supported by a specific and substantial utility that is credible or a well-established utility.

The arguments set forth above in Section VIII-A concerning the utility of the claimed receptors are herein incorporated in their entirety. Because claims 1, 7-10, 12-25, and 29-33 are supported by a specific and substantial utility that is credible and a well-established utility, the rejection under 35 U.S.C. §112, first paragraph, cannot stand and should, therefore, be overruled.

IX. CONCLUSION

The rejections of claims 1, 7-10, 12-25, and 29-33 under 35 U.S.C. §§ 101 and 112, first paragraph, are improper and should be reversed. For the reasons given above, appealed claims 1, 7-10, 12-25, and 29-33 are patentable.

Respectfully submitted,



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APPENDIX TO APPELLANT'S BRIEF PURSUANT TO 37 C.F.R. §1.192

Appendix A Claims on Appeal

Appendix B BLAST search results

Appendix C NCBI Sequence Viewer for NP_997055

Appendix D NCBI Sequence Viewer for NP_997056

Appendix E Laitinen et al., Characterization of a Common Susceptibility Locus for Asthma-Related Traits. Science, 2004, 304:300-304

Appendix F Entrez Gene report

Appendix G Shimuara *et al.*, Urinary Arginine Vasopressin in Asthma: Consideration of Fluid Therapy. Acta Paediatr. Jpn. 1990, 32(2) 197-200

Appendix H Thibonnier, Genetics of Vasopressin Receptors. Current Hypertension Reports, 2004, 6:21-26, Current Science, Inc. ISSN 1522-6417

Appendix I Thibonnier *et al.*, Molecular pharmacology of human vasopressin receptors. *Adv Exp Med Biol*, 1998, 449:251-276

The following claims are on appeal:

Claim 1 An isolated nucleic acid molecule comprising a nucleotide sequence that encodes a polypeptide comprising an amino acid sequence of SEQ ID NO: 25.

Claim 7 The isolated nucleic acid molecule of claim 1 wherein said nucleic acid molecule is DNA.

Claim 8 The isolated nucleic acid molecule of claim 1 wherein said nucleic acid molecule is RNA.

Claim 9 An expression vector comprising the nucleic acid molecule of claim 1.

Claim 10 The expression vector of claim 9 wherein said nucleic acid molecule comprises SEQ ID NO:12.

Claim 12 The expression vector of claim 9 wherein said vector is a plasmid.

Claim 13 The expression vector of claim 9 wherein said vector is a viral particle.

Claim 14 The expression vector of claim 13 wherein said vector is selected from the group consisting of adenoviruses, baculoviruses, parvoviruses, herpesviruses, poxviruses, adenoassociated viruses, Semlike Forest viruses, vaccinia viruses, and retroviruses.

Claim 15 The expression vector of claim 9 wherein said nucleic acid molecule is operably connected to a promoter selected from the group consisting of simian virus 40, mouse mammary tumor virus, long terminal repeat of human immunodeficiency virus, maloney virus,

cytomegalovirus immediate early promoter, Epstein Barr virus, rous sarcoma virus, human actin, human myosin, human hemoglobin, human muscle creatine, and human metalothionein.

Claim 16 A host cell transformed with the expression vector of claim 9.

Claim 17 The transformed host cell of claim 16 wherein said cell is a bacterial cell.

Claim 18 The transformed host cell of claim 17 wherein said bacterial cell is E. coli.

Claim 19 The transformed host cell of claim 16 wherein said cell is yeast.

Claim 20 The transformed host cell of claim 19 wherein said yeast is S. cerevisiae.

Claim 21 The transformed host cell of claim 16 wherein said cell is an insect cell.

Claim 22 The transformed host cell of claim 21 wherein said insect cell is S. frugiperda.

Claim 23 The transformed host cell of claim 16 wherein said cell is a mammalian cell.

Claim 24 The transformed host cell of claim 23 wherein said mammalian cell is selected from the group consisting of chinese hamster ovary cells, HeLa cells, African green monkey kidney cells, human 293 cells, and murine 3T3 fibroblasts.

Claim 25 An isolated nucleic acid molecule comprising SEQ ID NO:12.

Claim 29 A composition comprising a nucleic acid molecule of claim 1 or 25 and an acceptable carrier or diluent.

Claim 30 A composition comprising the expression vector of claim 9 and an acceptable carrier or diluent.

Claim 31 A method of producing a polypeptide that comprises SEQ ID NO:25, said method comprising the steps of:

a) introducing an expression vector of claim 10 into a compatible host cell;

b) growing said host cell under conditions for expression of said polypeptide; and

c) recovering said polypeptide.

Claim 32 The method of claim 31 wherein said host cell is lysed and said polypeptide is recovered from the lysate of said host cell.

Claim 33 The method of claim 31 wherein said polypeptide is recovered by purifying the culture medium without lysing said host cell.

NCBI **results of BLAST**

BLASTP 2.2.9 [May-01-2004]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1090247741-27419-36829700331.BLASTQ4

Query=
(371 letters)

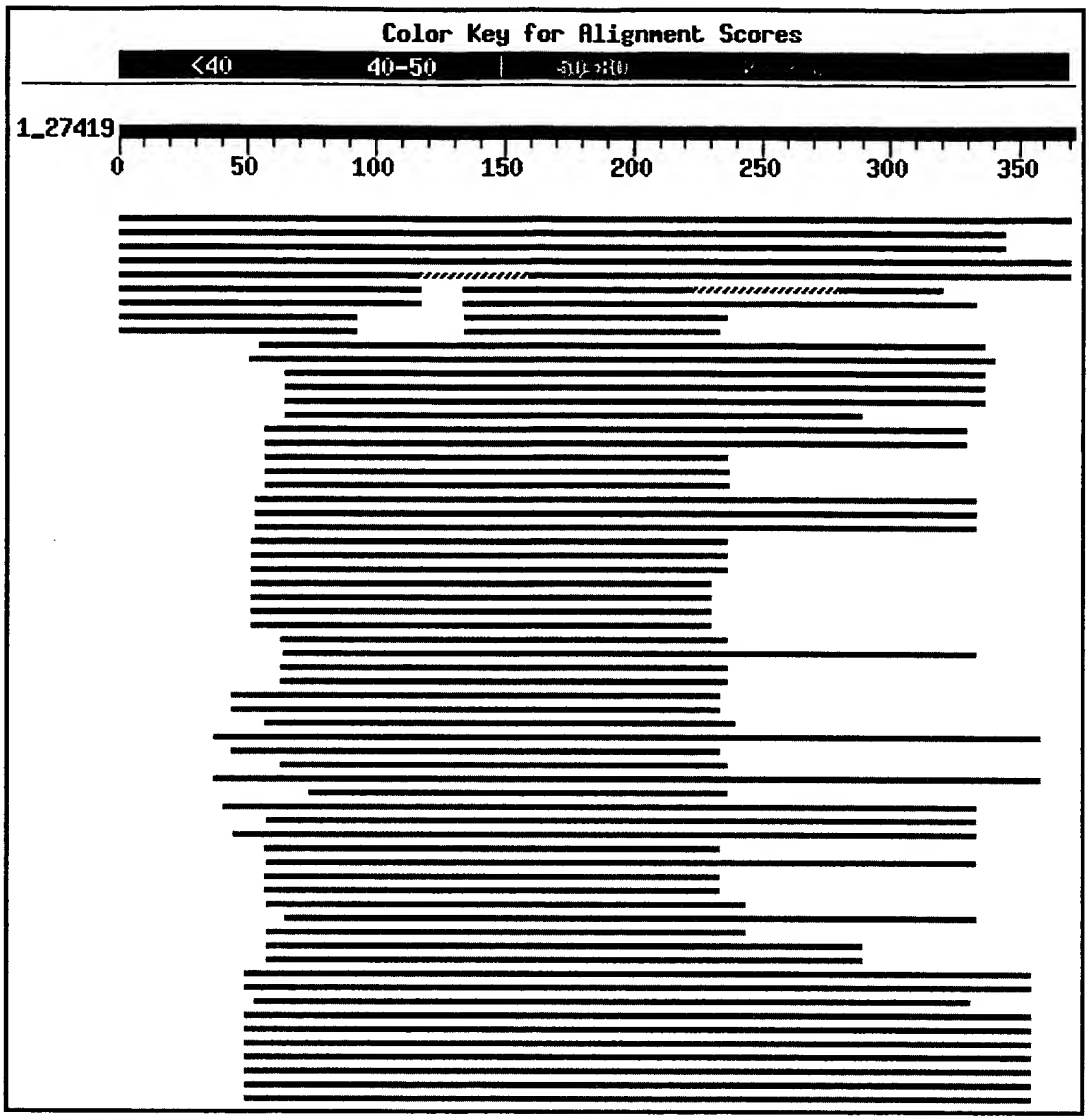
Database: All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples
1,933,177 sequences; 646,040,136 total letters

If you have any problems or questions with the results of this search please refer to the **BLAST FAQs**

Taxonomy reports

Distribution of 103 Blast Hits on the Query Sequence

Mouse-over to show defline and scores. Click to show alignments



Sequences producing significant alignments:	Score (bits)	E Value	
gi|46395496|ref|NP_997055.1| G protein-coupled receptor 154...	593	e-169	L
gi|46391085|ref|NP_997056.1| G protein-coupled receptor 154...	536	e-152	L
gi|34596868|gb|AAQ76968.1| GPRA isoform B short [Homo sapiens]	507	e-143	L
gi|42657892|ref|XP_376593.1| similar to RIKEN cDNA 9330128H...	453	e-127	L
gi|34596876|gb|AAQ76972.1| GPRA isoform F [Homo sapiens]	348	1e-95	L
gi|47077886|dbj|BAD18811.1| unnamed protein product [Homo s...	207	2e-53	
gi|34596874|gb|AAQ76971.1| GPRA isoform E [Homo sapiens]	204	3e-52	L
gi|34596872|gb|AAQ76970.1| GPRA isoform D [Homo sapiens]	166	9e-41	L
gi|34596870|gb|AAQ76969.1| GPRA isoform C [Homo sapiens]	153	4e-37	
gi|29611435|gb|AAO85048.1| G protein-coupled receptor PGR14...	140	4e-33	L
gi|4502333|ref|NP_000698.1| arginine vasopressin receptor 1...	102	1e-21	L
gi|4502331|ref|NP_000697.1| arginine vasopressin receptor 1...	101	2e-21	L
gi|228678|prf||1808301A oxytocin receptor	100	4e-21	

gi|266719|sp|P30559|OXYR_HUMAN Oxytocin receptor (OT-R) >gi...	100	5e-21	L
gi|32307152|ref|NP_000907.2| oxytocin receptor [Homo sapiens]	100	6e-21	L
gi|609015|emb|CAA56562.1| oxytocin receptor [Homo sapiens]	91	4e-18	L
gi|4557345|ref|NP_000045.1| arginine vasopressin receptor 2...	80	6e-15	L
gi|447224|prf||1913493A vasopressin receptor:ISOTYPE=V2	80	7e-15	
gi|2654031|gb|AAB87678.1| vasopressin receptor type 2 [Homo...	80	8e-15	L
gi|5453666|ref|NP_006134.1| G protein-coupled receptor 19 [...	75	1e-13	L
gi|7513259|pir||S74237 probable G protein-coupled receptor ...	75	1e-13	
gi|246909|gb|AAB21706.1| neurokinin-3 receptor; NK-3 recept...	72	1e-12	L
gi|7669548|ref|NP_001050.1| tachykinin receptor 3; NK-3 rec...	72	1e-12	L
gi|825695|emb|CAA46291.1| neuromedin K receptor [Homo sapiens]	72	1e-12	L
gi|21928727|dbj|BAC05950.1| seven transmembrane helix recep...	71	3e-12	L
gi|15281400|gb|AAK94199.1| neuropeptide NPVF receptor [Homo...	71	3e-12	L
gi|11545887|ref|NP_071429.1| G protein-coupled receptor 147...	71	3e-12	L
gi|13878604|sp|Q9Y5X5|NFF2_HUMAN Neuropeptide FF receptor 2...	69	1e-11	L
gi|16604258|ref|NP_444264.1| G protein-coupled receptor 74;...	69	1e-11	L
gi|4758820|ref|NP_004876.1| G protein-coupled receptor 74; ...	69	1e-11	L
gi|14279165|gb|AAK58513.1| G-protein-coupled receptor 74 [H...	69	2e-11	L
gi|4505513|ref|NP_000904.1| opiate receptor-like 1; rat LC1...	65	2e-10	L
gi|542922|pir||A44081 kappa-type opioid receptor - human >g...	65	2e-10	
gi|725266|gb|AAA96251.1| opioid receptor-like protein >gi|1...	65	3e-10	L
gi|49457117|emb|CAG46879.1| OPRL1 [Homo sapiens]	64	3e-10	
gi|4557859|ref|NP_001041.1| somatostatin receptor 2 [Homo s...	64	6e-10	L
gi|7229404|gb|AAF42810.1| somatostatin receptor 2B [Homo sa...	64	6e-10	L
gi|4557639|ref|NP_001517.1| orexin receptor 2 [Homo sapiens...	63	7e-10	L
gi|14550544|gb|AAH09522.1| Unknown (protein for IMAGE:33547...	63	9e-10	L
gi|28380053|sp|Q96P88|GRR2_HUMAN Gonadotropin-releasing hor...	63	1e-09	L
gi|18000079|gb|AAL54890.1| KOR-3D [Homo sapiens]	62	1e-09	L
gi|16589056|gb|AAL27000.1| type II gonadotropin-releasing h...	62	1e-09	L
gi|27435794|gb|AAO13224.1| KOR-3A splice variant [Homo sapi...	62	2e-09	L
gi|88267|pir||JQ1059 neurokinin 2 receptor - human >gi|1892...	62	2e-09	L
gi|21928731|dbj|BAC05952.1| seven transmembrane helix recep...	61	3e-09	
gi|4503905|ref|NP_003848.1| galanin receptor 2 [Homo sapien...	61	3e-09	L
gi|4507345|ref|NP_001048.1| tachykinin receptor 2; NK-2 rec...	61	4e-09	L
gi|11323189|emb|CAC17003.1| dJ1022E24.1 (opiate receptor-li...	61	4e-09	L
gi|19857032|sp|P21452|NK2R_HUMAN Substance-K receptor (SKR)...	60	4e-09	L
gi|27373028|gb|AAN87342.1| DRG kappa 1 splice variant KOR 1...	60	5e-09	L
gi|39725940|ref|NP_000903.2| opioid receptor, kappa 1; Opia...	60	6e-09	L
gi|730229|sp|P41145|OPRK_HUMAN Kappa-type opioid receptor (...	60	7e-09	L
gi|12652991|gb|AAH00256.1| Similar to somatostatin receptor...	59	9e-09	L
gi|4098212|gb|AAD00248.1| neuropeptide Y receptor type 2 [H...	59	1e-08	L
gi|238767|gb|AAB20304.1| substance K receptor, SK receptor...	59	1e-08	
gi|1000751|gb|AAA93170.1| type 2 neuropeptide Y receptor	59	2e-08	L
gi|4505447|ref|NP_000901.1| neuropeptide Y receptor Y2 [Hom...	59	2e-08	L
gi|15911833|gb|AAK38351.1| CCK-B/gastrin receptor variant [...	59	2e-08	L
gi|21426829|ref|NP_658986.1| G protein-coupled receptor 73-...	58	2e-08	L
gi|7688218|emb|CAB89854.1| dJ680N4.3 (novel G-protein coupl...	58	2e-08	L

gi|7690036|gb|AAB30766.2|	cholecystokinin B receptor [Homo ...	58	3e-08
gi|28875799|ref|NP_795344.1|	cholecystokinin B receptor; CC...	58	3e-08
gi|189232|gb|AAA59936.1|	neurokinin 1 receptor	57	4e-08
gi|7677460|gb|AAF67174.1|	CCK-B/gastrin receptor [Homo sapi...	57	4e-08
gi|3342090|gb|AAC27510.1|	gastrin\cholecystokinin brain rec...	57	5e-08
gi|9944990|gb|AAG03064.1|	neuromedin U receptor-type 2 [Hom...	57	5e-08
gi|4507343|ref|NP_001049.1|	tachykinin receptor 1 isoform 1...	57	5e-08
gi|9082156|gb|AAF82755.1|	neuromedin U receptor 2 [Homo sap...	57	5e-08
gi|20373179|ref|NP_620414.1|	G protein-coupled receptor 73;...	57	7e-08
gi|21327025|gb|AAM48127.1|	prokineticin receptor 1 [Homo sa...	57	7e-08
gi|22658465|gb|AAN01267.1|	beta 2 adrenergic receptor [Homo...	57	7e-08
gi|7669546|ref|NP_056542.1|	tachykinin receptor 1 isoform s...	56	8e-08
gi|4502819|ref|NP_000731.1|	cholinergic receptor, muscarini...	56	9e-08
gi|7706103|ref|NP_057652.1|	G-protein coupled receptor SALP...	56	9e-08
gi|2570533|gb|AAB82151.1|	beta2-adrenergic receptor [Homo s...	56	1e-07
gi|14573541|gb|AAK68114.1|	m3 muscarinic cholinergic recept...	56	1e-07
gi|1220299|gb|AAA91831.1|	cholecystokinin B receptor	56	1e-07
gi|5714688|gb|AAD48036.1|	beta-2-adrenergic receptor [Homo ...	56	1e-07
gi|19923823|ref|NP_064552.2|	neuromedin U receptor 2 [Homo ...	55	2e-07
gi|2570531|gb|AAB82150.1|	beta2-adrenergic receptor [Homo s...	55	2e-07
gi|4501969|ref|NP_000015.1|	adrenergic, beta-2-, receptor, ...	55	2e-07
gi|39645303|gb|AAH63486.1|	Unknown (protein for IMAGE:52417...	55	2e-07
gi|6573153|gb|AAF17569.1|	beta-2 andrenergic receptor [Homo...	55	2e-07
gi|178204|gb|AAA88017.1|	beta-2 adrenergic receptor >gi|257...	55	2e-07
gi|11095821|gb|AAG30036.1|	m3 muscarinic acetylcholine rece...	55	2e-07
gi|10835175|ref|NP_000612.1|	5-hydroxytryptamine (serotonin...	55	2e-07
gi|34190269|gb|AAH12481.2|	ADRB2 protein [Homo sapiens]	55	2e-07
gi|177776|gb|AAA58354.1|	serotonin receptor	55	2e-07
gi|88173|pir||JT0530	muscarinic acetylcholine receptor M5 -...	55	3e-07
gi|1314330|gb|AAB07760.1|	neuropeptide y/peptide YY recepto...	54	3e-07
gi|2570529|gb|AAB82149.1|	beta2-adrenergic receptor [Homo s...	54	3e-07
gi|29373|emb|CAA28511.1|	unnamed protein product [Homo sapi...	54	3e-07
gi|4504531|ref|NP_000515.1|	5-hydroxytryptamine (serotonin)...	54	4e-07
gi|405310|gb|AAB26819.1|	D2 dopamine receptor [Homo sapiens]	54	4e-07
gi|7108336|ref|NP_036257.1|	cholinergic receptor, muscarini...	54	5e-07
gi|4502607|ref|NP_000721.1|	cholecystokinin A receptor [Hom...	54	5e-07
gi|27469809|gb|AAH41805.1|	Cholinergic receptor, muscarinic...	54	5e-07
gi|14573545|gb|AAK68116.1|	m5 muscarinic cholinergic recept...	54	5e-07
gi|1737179|gb|AAC14587.1|	somatostatin receptor-like protei...	54	5e-07
gi|225717|prf||1311340A	G protein coupled receptor	54	5e-07

Alignments

Get selected sequences | Select all | Deselect all

>gi|46395496|ref|NP_997055.1| G protein-coupled receptor 154 isoform A; G prot
receptor for asthma susceptibility; vasopressin
receptor-related receptor 1 [Homo sapiens]
gi|34596864|gb|AAQ76966.1| GPRA isoform A [Homo sapiens]
Length = 371

Score = 593 bits (1529), Expect = e-169
Identities = 314/371 (84%), Positives = 314/371 (84%)

```
Query: 1   MPANFTEGSFDSSGTGQTLDSSPVACXXXXXXXXXXXXGKEWGSFYYSFKTEQLITLWVLF 60
           MPANFTEGSFDSSGTGQTLDSSPVAC           GKEWGSFYYSFKTEQLITLWVLF
Sbjct: 1   MPANFTEGSFDSSGTGQTLDSSPVACTETVTFTEVVEGKEWGSFYYSFKTEQLITLWVLF 60

Query: 61  VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTAPDXX 120
           VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNILTDI WRFTGDFTAPD
Sbjct: 61  VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNILTDINWRFTGDFTAPDLV 120

Query: 121 XXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIP 180
                        ASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIP
Sbjct: 121 CRVVRYLQVVLLYASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIP 180

Query: 181 TLIIFGKRTLSNGEVQCWALWPGDSYWTPYMTIVAFLVYFIPLTIISIMYGIVIRTIWIK 240
           TLIIFGKRTLSNGEVQCWALWP DSYWTPYMTIVAFLVYFIPLTIISIMYGIVIRTIWIK
Sbjct: 181 TLIIFGKRTLSNGEVQCWALWPDDSYWTPYMTIVAFLVYFIPLTIISIMYGIVIRTIWIK 240

Query: 241 SKTYETVISNCSDGKLCSSYNRGLXXXXXXXXXXXXXXXXLAFICCWSPYXXXXXXXXXX 300
           SKTYETVISNCSDGKLCSSYNRGL                 LAFICCWSPY
Sbjct: 241 SKTYETVISNCSDGKLCSSYNRGLISKAKIKAIKYSIIIILAFICCWSPYFLFDILDNFN 300

Query: 301 XXPDTQERFYASVIIQNLPALNSAINPPIYCVFSSSISFPCREQRSQDSRMTFRERTERH 360
             PDTQERFYASVIIQNLPALNSAINP IYCVFSSSISFPCREQRSQDSRMTFRERTERH
Sbjct: 301 LLPDTQERFYASVIIQNLPALNSAINPLIYCVFSSSISFPCREQRSQDSRMTFRERTERH 360

Query: 361 EMQILSKPEFI 371
           EMQILSKPEFI
Sbjct: 361 EMQILSKPEFI 371
```

>gi|46391085|ref|NP_997056.1| G protein-coupled receptor 154 isoform B; G prot
receptor for asthma susceptibility; vasopressin
receptor-related receptor 1 [Homo sapiens]
gi|34596866|gb|AAQ76967.1| GPRA isoform B long [Homo sapiens]
Length = 377

Score = 536 bits (1380), Expect = e-152
Identities = 286/345 (82%), Positives = 286/345 (82%)

```
Query: 1   MPANFTEGSFDSSGTGQTLDSSPVACXXXXXXXXXXXXGKEWGSFYYSFKTEQLITLWVLF 60
           MPANFTEGSFDSSGTGQTLDSSPVAC           GKEWGSFYYSFKTEQLITLWVLF
Sbjct: 1   MPANFTEGSFDSSGTGQTLDSSPVACTETVTFTEVVEGKEWGSFYYSFKTEQLITLWVLF 60

Query: 61  VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTAPDXX 120
           VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNILTDI WRFTGDFTAPD
Sbjct: 61  VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNILTDINWRFTGDFTAPDLV 120

Query: 121 XXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIP 180
                        ASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIP
Sbjct: 121 CRVVRYLQVVLLYASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIP 180
```

```
Query: 181 TLIIFGKRTLSNGEVQCWALWPGDSYWTPYMTIVAFLVYFIPLTIISIMYGIVIRTIWIK 240
           TLIIFGKRTLSNGEVQCWALWP DSYWTPYMTIVAFLVYFIPLTIISIMYGIVIRTIWIK
Sbjct: 181 TLIIFGKRTLSNGEVQCWALWPDDSYWTPYMTIVAFLVYFIPLTIISIMYGIVIRTIWIK 240

Query: 241 SKTYETVISNCSDGKLCSSYNRGLXXXXXXXXXXXXXXXXLAFICCWSPYXXXXXXXXXX 300
           SKTYETVISNCSDGKLCSSYNRGL                LAFICCWSPY
Sbjct: 241 SKTYETVISNCSDGKLCSSYNRGLISKAKIKAIKYSIIIILAFICCWSPYFLFDILDNFN 300

Query: 301 XXPDTQERFYASVIIQNLPALNSAINPPIYCVFSSSISFPCREQR 345
             PDTQERFYASVIIQNLPALNSAINP IYCVFSSSISFPCR  R
Sbjct: 301 LLPDTQERFYASVIIQNLPALNSAINPLIYCVFSSSISFPCRVIR 345
```

>gi|34596868|gb|AAQ76968.1| GPRA isoform B short [Homo sapiens]
Length = 366

```
 Score =  507 bits (1306), Expect = e-143
 Identities = 275/345 (79%), Positives = 275/345 (79%), Gaps = 11/345 (3%)

Query: 1   MPANFTEGSFDSSGTGQTLDSSPVACXXXXXXXXXXXGKEWGSFYYSFKTEQLITLWVLF 60
           MPANFTEGSFDSSGTGQTLDSSPVAC           GKEWGSFYYSFKTEQLITLWVLF
Sbjct: 1   MPANFTEGSFDSSGTGQTLDSSPVACTETVTFTEVVEGKEWGSFYYSFKTEQLITLWVLF 60

Query: 61  VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTAPDXX 120
           VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITD           I WRFTGDFTAPD
Sbjct: 61  VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITD-----------INWRFTGDFTAPDLV 109

Query: 121 XXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIP 180
                       ASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIP
Sbjct: 110 CRVVRYLQVVLLYASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIP 169

Query: 181 TLIIFGKRTLSNGEVQCWALWPGDSYWTPYMTIVAFLVYFIPLTIISIMYGIVIRTIWIK 240
           TLIIFGKRTLSNGEVQCWALWP DSYWTPYMTIVAFLVYFIPLTIISIMYGIVIRTIWIK
Sbjct: 170 TLIIFGKRTLSNGEVQCWALWPDDSYWTPYMTIVAFLVYFIPLTIISIMYGIVIRTIWIK 229

Query: 241 SKTYETVISNCSDGKLCSSYNRGLXXXXXXXXXXXXXXXXLAFICCWSPYXXXXXXXXXX 300
           SKTYETVISNCSDGKLCSSYNRGL                LAFICCWSPY
Sbjct: 230 SKTYETVISNCSDGKLCSSYNRGLISKAKIKAIKYSIIIILAFICCWSPYFLFDILDNFN 289

Query: 301 XXPDTQERFYASVIIQNLPALNSAINPPIYCVFSSSISFPCREQR 345
             PDTQERFYASVIIQNLPALNSAINP IYCVFSSSISFPCR  R
Sbjct: 290 LLPDTQERFYASVIIQNLPALNSAINPLIYCVFSSSISFPCRVIR 334
```

>gi|42657892|ref|XP_376593.1| similar to RIKEN cDNA 9330128H10 gene [Homo sap:
gi|42658273|ref|XP_379807.1| similar to RIKEN cDNA 9330128H10 gene [Homo sapien
Length = 522

```
 Score =  453 bits (1165), Expect = e-127
 Identities = 232/278 (83%), Positives = 232/278 (83%)

Query: 94  DSFTGLVNILTDIIWRFTGDFTAPDXXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYP 153
           DSFTGLVNILTDI WRFTGDFTAPD                ASTYVLVSLSIDRYHAIVYP
Sbjct: 245 DSFTGLVNILTDINWRFTGDFTAPDLVCRVVRYLQVVLLYASTYVLVSLSIDRYHAIVYP 304

Query: 154 MKFLQGEKQARVLIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQCWALWPGDSYWTPYMTI 213
```

```
            MKFLQGEKQARVLIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQCWALWP DSYWTPYMTI
Sbjct: 305 MKFLQGEKQARVLIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQCWALWPDDSYWTPYMTI 364

Query: 214 VAFLVYFIPLTIISIMYGIVIRTIWIKSKTYETVISNCSDGKLCSSYNRGLXXXXXXXXX 273
            VAFLVYFIPLTIISIMYGIVIRTIWIKSKTYETVISNCSDGKLCSSYNRGL
Sbjct: 365 VAFLVYFIPLTIISIMYGIVIRTIWIKSKTYETVISNCSDGKLCSSYNRGLISKAKIKAI 424

Query: 274 XXXXXXXLAFICCWSPYXXXXXXXXXXXXXPDTQERFYASVIIQNLPALNSAINPPIYCVF 333
                  LAFICCWSPY            PDTQERFYASVIIQNLPALNSAINP IYCVF
Sbjct: 425 KYSIIIILAFICCWSPYFLFDILDNFNLLPDTQERFYASVIIQNLPALNSAINPLIYCVF 484

Query: 334 SSSISFPCREQRSQDSRMTFRERTERHEMQILSKPEFI 371
            SSSISFPCREQRSQDSRMTFRERTERHEMQILSKPEFI
Sbjct: 485 SSSISFPCREQRSQDSRMTFRERTERHEMQILSKPEFI 522


 Score =  162 bits (410), Expect = 1e-39
 Identities = 82/93 (88%), Positives = 82/93 (88%)

Query: 1  MPANFTEGSFDSSGTGQTLDSSPVACXXXXXXXXXXXXGKEWGSFYYSFKTEQLITLWVLF 60
           MPANFTEGSFDSSGTGQTLDSSPVAC           GKEWGSFYYSFKTEQLITLWVLF
Sbjct: 1  MPANFTEGSFDSSGTGQTLDSSPVACTETVTFTEVVEGKEWGSFYYSFKTEQLITLWVLF 60

Query: 61 VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAIT 93
           VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAIT
Sbjct: 61 VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAIT 93


>gi|34596876|gb|AAQ76972.1| GPRA isoform F [Homo sapiens]
          Length = 305

 Score =  348 bits (892), Expect = 1e-95
 Identities = 182/212 (85%), Positives = 182/212 (85%)

Query: 160 EKQARVLIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQCWALWPGDSYWTPYMTIVAFLVY 219
            EKQARVLIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQCWALWP DSYWTPYMTIVAFLVY
Sbjct: 94  EKQARVLIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQCWALWPDDSYWTPYMTIVAFLVY 153

Query: 220 FIPLTIISIMYGIVIRTIWIKSKTYETVISNCSDGKLCSSYNRGLXXXXXXXXXXXXXXX 279
            FIPLTIISIMYGIVIRTIWIKSKTYETVISNCSDGKLCSSYNRGL
Sbjct: 154 FIPLTIISIMYGIVIRTIWIKSKTYETVISNCSDGKLCSSYNRGLISKAKIKAIKYSIII 213

Query: 280 XLAFICCWSPYXXXXXXXXXXXXXPDTQERFYASVIIQNLPALNSAINPPIYCVFSSSISF 339
             LAFICCWSPY            PDTQERFYASVIIQNLPALNSAINP IYCVFSSSISF
Sbjct: 214 ILAFICCWSPYFLFDILDNFNLLPDTQERFYASVIIQNLPALNSAINPLIYCVFSSSISF 273

Query: 340 PCREQRSQDSRMTFRERTERHEMQILSKPEFI 371
            PCREQRSQDSRMTFRERTERHEMQILSKPEFI
Sbjct: 274 PCREQRSQDSRMTFRERTERHEMQILSKPEFI 305


 Score =  159 bits (402), Expect = 7e-39
 Identities = 87/117 (74%), Positives = 91/117 (77%), Gaps = 3/117 (2%)

Query: 1   MPANFTEGSFDSSGTGQTLDSSPVACXXXXXXXXXXXXGKEWGSFYYSFKTEQLITLWVLF 60
            MPANFTEGSFDSSGTGQTLDSSPVAC           GKEWGSFYYSFKTEQLITLWVLF
Sbjct: 1   MPANFTEGSFDSSGTGQTLDSSPVACTETVTFTEVVEGKEWGSFYYSFKTEQLITLWVLF 60
```

```
Query: 61  VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTAP 117
           VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAIT+       +L  I W  +  F+ P
Sbjct: 61  VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITEK---QARVLIVIAWSLSFLFSIP 114
```

>gi|47077886|dbj|BAD18811.1| unnamed protein product [Homo sapiens]
Length = 143

Score = 207 bits (528), Expect = 2e-53
Identities = 107/118 (90%), Positives = 107/118 (90%)

```
Query: 1   MPANFTEGSFDSSGTGQTLDSSPVACXXXXXXXXXXXGKEWGSFYYSFKTEQLITLWVLF 60
           MPANFTEGSFDSSGTGQTLDSSPVAC           GKEWGSFYYSFKTEQLITLWVLF
Sbjct: 1   MPANFTEGSFDSSGTGQTLDSSPVACTETVTFTEVVEGKEWGSFYYSFKTEQLITLWVLF 60

Query: 61  VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTAPD 118
           VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTAPD
Sbjct: 61  VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTAPD 118
```

>gi|34596874|gb|AAQ76971.1| GPRA isoform E [Homo sapiens]
Length = 136

Score = 204 bits (518), Expect = 3e-52
Identities = 106/118 (89%), Positives = 106/118 (89%)

```
Query: 1   MPANFTEGSFDSSGTGQTLDSSPVACXXXXXXXXXXXGKEWGSFYYSFKTEQLITLWVLF 60
           MPANFTEGSFDSSGTGQTLDSSPVAC           GKEWGSFYYSFKTEQLITLWVLF
Sbjct: 1   MPANFTEGSFDSSGTGQTLDSSPVACTETVTFTEVVEGKEWGSFYYSFKTEQLITLWVLF 60

Query: 61  VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTAPD 118
           VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNILTDI WRFTGDFTAPD
Sbjct: 61  VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNILTDINWRFTGDFTAPD 118
```

>gi|34596872|gb|AAQ76970.1| GPRA isoform D [Homo sapiens]
Length = 158

Score = 166 bits (419), Expect = 9e-41
Identities = 82/93 (88%), Positives = 82/93 (88%)

```
Query: 1   MPANFTEGSFDSSGTGQTLDSSPVACXXXXXXXXXXXGKEWGSFYYSFKTEQLITLWVLF 60
           MPANFTEGSFDSSGTGQTLDSSPVAC           GKEWGSFYYSFKTEQLITLWVLF
Sbjct: 1   MPANFTEGSFDSSGTGQTLDSSPVACTETVTFTEVVEGKEWGSFYYSFKTEQLITLWVLF 60

Query: 61  VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAIT 93
           VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAIT
Sbjct: 61  VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAIT 93
```

>gi|34596870|gb|AAQ76969.1| GPRA isoform C [Homo sapiens]
Length = 94

Score = 153 bits (387), Expect = 4e-37

```
 Identities = 82/93 (88%), Positives = 82/93 (88%)

Query: 1  MPANFTEGSFDSSGTGQTLDSSPVACXXXXXXXXXXXGKEWGSFYYSFKTEQLITLWVLF 60
          MPANFTEGSFDSSGTGQTLDSSPVAC           GKEWGSFYYSFKTEQLITLWVLF
Sbjct: 1  MPANFTEGSFDSSGTGQTLDSSPVACTETVTFTEVVEGKEWGSFYYSFKTEQLITLWVLF 60

Query: 61 VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAIT 93
          VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAIT
Sbjct: 61 VFTIVGNSVVLFSTWRRKKKSRMTFFVTQLAIT 93
```

>gi|29611435|gb|AAO85048.1| G protein-coupled receptor PGR14 [Homo sapiens]
Length = 129

```
 Score =  140 bits (353), Expect = 4e-33
 Identities = 76/90 (84%), Positives = 76/90 (84%), Gaps = 12/90 (13%)

Query: 134 ASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIPTLIIFGKRTLSNG 193
           ASTYVLVSLSIDRYHAIVYPM            IVIAWSLSFLFSIPTLIIFGKRTLSNG
Sbjct: 14  ASTYVLVSLSIDRYHAIVYPM------------IVIAWSLSFLFSIPTLIIFGKRTLSNG 61

Query: 194 EVQCWALWPGDSYWTPYMTIVAFLVYFIPL 223
           EVQCWALWP DSYWTPYMTIVAFLVYFIP
Sbjct: 62  EVQCWALWPDDSYWTPYMTIVAFLVYFIPF 91
```

```
 Score = 56.6 bits (135), Expect = 7e-08
 Identities = 27/41 (65%), Positives = 28/41 (68%)

Query: 281 LAFICCWSPYXXXXXXXXXXXXPDTQERFYASVIIQNLPAL 321
           + FICCWSPY           PDTQERFYASVIIQNLPAL
Sbjct: 89  IPFICCWSPYFLFDILDNFNLLPDTQERFYASVIIQNLPAL 129
```

>gi|4502333|ref|NP_000698.1| arginine vasopressin receptor 1B; arginine vasopi
antidiuretic hormone receptor 1B; vasopressin V1B
receptor; pituitary vasopressin receptor 3 [Homo
sapiens]
gi|1351392|sp|P47901|V1BR_HUMAN Vasopressin V1b receptor (V1bR) (AVPR V1b) (Vas
receptor) (AVPR V3) (Antidiuretic hormone receptor 1b)
gi|627665|pir||A55089 vasopressin V3 receptor - human
gi|563982|dbj|BAA06621.1| Vasopressin V1b Receptor [Homo sapiens]
gi|722622|gb|AAA65687.1| vasopressin V3 receptor
gi|2613125|gb|AAB84293.1| small cell vasopressin subtype 1b receptor [Homo sapi
gi|4336682|gb|AAD17892.1| vasopressin receptor subtype 1b [Homo sapiens]
gi|6969253|gb|AAF33681.1| V1b vasopressin receptor [Homo sapiens]
gi|1092970|prf||2102273A vasopressin receptor
Length = 424

```
 Score =  102 bits (253), Expect = 1e-21
 Identities = 84/307 (27%), Positives = 134/307 (43%), Gaps = 27/307 (8%)

Query: 56  LWVLFVFTIVGNSVVLFSTWRR-KKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDF 114
           L  + V    GN  VL +  +  +K+SRM  FV  LA+TD    L  +L  ++W  T  F
```

```
Sbjct: 41  LATVLVLATGGNLAVLLTLGQLGRKRSRMHLFVLHLALTDLAVALFQVLPQLLWDITYRF 100

Query: 115 TAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIA-WSL 173
             PD               ASTY+L+++++DRY A+ +P++ LQ   Q+  L++ A W L
Sbjct: 101 QGPDLLCRAVKYLQVLSMFASTYMLLAMTLDRYLAVCHPLRSLQQPGQSTYLLIAAPWLL 160

Query: 174 SFLFSIPTLIIFGKRTL--SNGEVQCWALWPGDSYWTP--YMTIVAFLVYFIPLTIISIM 229
           + +FS+P + IF  R +   +G + CWA +     W P  Y+T     ++ +P+T+++
Sbjct: 161 AAIFSLPQVFIFSLREVIQGSGVLDCWADF--GFPWGPRAYLTWTTLAIFVLPVTMLTAC 218

Query: 230 YGIVIRTIW--IKSKTYETVIS-------NCSDGKLCSSYNRGL---------XXXXXXX 271
           Y ++   I   +K KT    +         +       ++  RGL
Sbjct: 219 YSLICHEICKNLKVKTQAWRVGGGGWRTWDRPSPSTLAATTRGLPSRVSSINTISRAKIR 278

Query: 272 XXXXXXXXXLAFICCWSP-YXXXXXXXXXXXXPDTQERFYASVIIQNLPALNSAINPPIY 330
                  LA+I CW+P +             PD      A  I   L  LNS  NP IY
Sbjct: 279 TVKMTFVIVLAYIACWAPFFSVQMWSVWDKNAPDEDSTNVAFTISMLLGNLNSCCNPWIY 338

Query: 331 CVFSSSI 337
             F+S +
Sbjct: 339 MGFNSHL 345
```

☑ >gi|4502331|ref|NP_000697.1| L arginine vasopressin receptor 1A; V1a vasopressir
vascular/hepatic-type arginine vasopressin receptor;
antidiuretic hormone receptor 1A; V1-vascular
vasopressin receptor AVPR1A; SCCL vasopressin subtype 1a
receptor [Homo sapiens]

gi|586197|sp|P37288|V1AR_HUMAN L Vasopressin V1a receptor (V1aR) (Vascular/hepati
vasopressin receptor) (Antidiuretic hormone receptor 1a)
(AVPR V1a)

gi|1082914|pir||A53046 vasopressin receptor V1a - human

gi|667068|gb|AAA62271.1| L arginine vasopressin receptor 1

gi|755464|gb|AAB19232.1| L arginine vasopressin receptor 1

gi|786550|gb|AAC60638.1| L vascular-type vasopressin receptor; V1 vasopressin rec
sapiens]

gi|2623230|gb|AAC51861.1| L SCCL vasopressin subtype 1a receptor [Homo sapiens]

gi|4336680|gb|AAD17891.1| L vasopressin receptor subtype 1a [Homo sapiens]

gi|32482021|gb|AAP84363.1| L arginine vasopressin receptor 1A [Homo sapiens]

gi|1588287|prf||2208303A vasopressin receptor V1a

Length = 418

Score = 101 bits (251), Expect = 2e-21
Identities = 79/312 (25%), Positives = 127/312 (40%), Gaps = 22/312 (7%)

```
Query: 52  QLITLWVLFVFTIVGNSVVLFSTWRR-KKKSRMTFFVTQLAITDSFTGLVNILTDIIWRF 110
           ++  L V F   ++GNS VL +  R  +K SRM  F+  L++ D       +L  + W
Sbjct: 54  EIAVLAVTFAVAVLGNSSVLLALHRTPRKTSRMHLFIRHLSLADLAVAFFQVLPQMCWDI 113

Query: 111 TGDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQG-EKQARVLIVI 169
           T  F  PD               AS Y+LV ++ DRY A+ +P+K LQ   +++R++I
Sbjct: 114 TYRFRGPDWLCRVVKHLQVFGMFASAYMLVVMTADRYIAVCHPLKTLQQPARRSRLMIAA 173

Query: 170 AWSLSFLFSIPTLIIFGKRTLSN--GEVQCWALWPGDSYWTPYMTIVAFLVYFIPLTIIS 227
           AW LSF+ S P   +F    ++N      CWA +         Y+T +   ++  P+ I+
Sbjct: 174 AWVLSFVLSTPQYFVFSMIEVNNVTKARDCWATFIQPWGSRAYVTWMTGGIFVAPVVILG 233
```

```
Query: 228 IMYGIVIRTIWIKSKTYETVISNCSDGKLCSSYNRGLXXXXXXXXXXXXXXXXL------ 281
            YG +   IW   +       +    +   ++ +G                 +
Sbjct: 234 TCYGFICYNIWCNVRGKTASRQSKGAEQAGVAFQKGFLLAPCVSSVKSISRAKIRTVKMT 293

Query: 282 -----AFICCWSPYXXXXXXXXXXXXPDTQERFYASVIIQN-LPALNSAINPPIYCVFSS 335
                A+I CW+P+                  E    ++ I   L +LNS  NP IY  FS
Sbjct: 294 FVIVTAYIVCWAPFFIIQMWSVWDPMSVWTESENPTITITALLGSLNSCCNPWIYMFFSG 353

Query: 336 SI------SFPC 341
            +      SFPC
Sbjct: 354 HLLQDCVQSFPC 365
```

☑ >gi|228678|prf||1808301A oxytocin receptor

Length = 388

```
 Score =  100 bits (249), Expect = 4e-21
 Identities = 77/285 (27%), Positives = 128/285 (44%), Gaps = 18/285 (6%)

Query: 66  GNSVVLFS-TWRRKKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTAPDXXXXXX 124
           GN+ VL +    R+K SR+ FF+  L+I D    +  +L  ++W  T  F  PD
Sbjct: 56  GNACVLLALRTTRQKHSRLFFFMKHLSIADLVVAVFQVLPQLLWDITFRFYGPDLLCRLV 115

Query: 125 XXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIPTLII 184
                    ASTY+L+ +S+DR  AI  P++ L+  +  R+ ++  W    + S P + I
Sbjct: 116 KYLQVVGMFASTYLLLLMSLDRCLAICQPLRSLR-RRTDRLAVLATWLGCLVASAPQVHI 174

Query: 185 FGKRTLSNGEVQCWALWPGDSYWTP--YMTIVAFLVYFIPLTIISIMYGIVIRTIW--IK 240
           F  R +++G   CWA++     W P  Y+T +   VY +P+ +++  YG++   IW  ++
Sbjct: 175 FSLREVADGVFDCWAVFI--QPWGPKAYITWITLAVYIVPVIVLATCYGLISFKIWQNLR 232

Query: 241 SKT-----YETVISNCSDG---KLCSSYNRGLXXXXXXXXXXXXXXXXLAFICCWSPYXX 292
            KT          +   DG    L    +  L                LAFI CW+P+
Sbjct: 233 LKTAAAGAEAPEGAAAGDGGRVALARVSSVKLISKAKIRTVKMTFIIVLAFIVCWTPF-- 290

Query: 293 XXXXXXXXXXPDTQERFYASVIIQNLPALNSAINPPIYCVFSSSI 337
                     +  +   A +I+  L +LNS  NP IY +F+  +
Sbjct: 291 FFVQMWSVWDANAPKEASAFIIVMLLASLNSCCNPWIYMLFTGHL 335
```

☑ >gi|266719|sp|P30559|OXYR_HUMAN L Oxytocin receptor (OT-R)
gi|1082671|pir||A55493 oxytocin receptor - human
gi|34765|emb|CAA46097.1| L oxytocin receptor [Homo sapiens]
gi|37362417|gb|AAQ91333.1| L oxytocin receptor [Homo sapiens]

Length = 389

```
 Score =  100 bits (248), Expect = 5e-21
 Identities = 77/286 (26%), Positives = 128/286 (44%), Gaps = 19/286 (6%)

Query: 66  GNSVVLFS-TWRRKKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTAPDXXXXXX 124
           GN+ VL +    R+K SR+ FF+  L+I D    +  +L  ++W  T  F  PD
Sbjct: 56  GNACVLLALRTTRQKHSRLFFFMKHLSIADLVVAVFQVLPQLLWDITFRFYGPDLLCRLV 115

Query: 125 XXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIPTLII 184
                    ASTY+L+ +S+DR  AI  P++ L+  +  R+ ++  W    + S P + I
Sbjct: 116 KYLQVVGMFASTYLLLLMSLDRCLAICQPLRSLR-RRTDRLAVLATWLGCLVASAPQVHI 174
```

```
Query: 185 FGKRTLSNGEVQCWALWPGDSYWTP--YMTIVAFLVYFIPLTIISIMYGIVIRTIW--IK 240
           F  R +++G   CWA++     W P  Y+T +   VY +P+ +++  YG++   IW  ++
Sbjct: 175 FSLREVADGVFDCWAVFI--QPWGPKAYITWITLAVYIVPVIVLATCYGLISFKIWQNLR 232

Query: 241 SKT------YETVISNCSDG---KLCSSYNRGLXXXXXXXXXXXXXXXXLAFICCWSPYX 291
            KT           +   DG    L    +  L                LAFI CW+P+
Sbjct: 233 LKTAAAAAAEAPEGAAAGDGGRVALARVSSVKLISKAKIRTVKMTFIIVLAFIVCWTPF- 291

Query: 292 XXXXXXXXXXXPDTQERFYASVIIQNLPALNSAINPPIYCVFSSSI 337
                      +  +   A +I+  L +LNS  NP IY +F+  +
Sbjct: 292 -FFVQMWSVWDANAPKEASAFIIVMLLASLNSCCNPWIYMLFTGHL 336
```

>gi|32307152|ref|NP_000907.2| oxytocin receptor [Homo sapiens]
Length = 389

```
 Score =  100 bits (248), Expect = 6e-21
 Identities = 77/286 (26%), Positives = 128/286 (44%), Gaps = 19/286 (6%)

Query: 66  GNSVVLFS-TWRRKKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTAPDXXXXXX 124
           GN+ VL +    R+K SR+ FF+  L+I D    +  +L  ++W  T  F  PD
Sbjct: 56  GNACVLLALRTTRQKHSRLFFFMKHLSIADLVVAVFQVLPQLLWDITFRFYGPDLLCRLV 115

Query: 125 XXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIPTLII 184
                  ASTY+L+ +S+DR  AI  P++ L+  +  R+ ++  W    + S P + I
Sbjct: 116 KYLQVVGMFASTYLLLLMSLDRCLAICQPLRSLR-RRTDRLAVLATWLGCLVASAPQVHI 174

Query: 185 FGKRTLSNGEVQCWALWPGDSYWTP--YMTIVAFLVYFIPLTIISIMYGIVIRTIW--IK 240
           F  R +++G   CWA++     W P  Y+T +   VY +P+ +++  YG++   IW  ++
Sbjct: 175 FSLREVADGVFDCWAVFI--QPWGPKAYITWITLAVYIVPVIVLAACYGLISFKIWQNLR 232

Query: 241 SKT------YETVISNCSDG---KLCSSYNRGLXXXXXXXXXXXXXXXXLAFICCWSPYX 291
            KT           +   DG    L    +  L                LAFI CW+P+
Sbjct: 233 LKTAAAAAAEAPEGAAAGDGGRVALARVSSVKLISKAKIRTVKMTFIIVLAFIVCWTPF- 291

Query: 292 XXXXXXXXXXXPDTQERFYASVIIQNLPALNSAINPPIYCVFSSSI 337
                      +  +   A +I+  L +LNS  NP IY +F+  +
Sbjct: 292 -FFVQMWSVWDANAPKEASAFIIVMLLASLNSCCNPWIYMLFTGHL 336
```

>gi|609015|emb|CAA56562.1| oxytocin receptor [Homo sapiens]
Length = 308

```
 Score = 90.5 bits (223), Expect = 4e-18
 Identities = 66/239 (27%), Positives = 109/239 (45%), Gaps = 17/239 (7%)

Query: 66  GNSVVLFS-TWRRKKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTAPDXXXXXX 124
           GN+ VL +    R+K SR+ FF+  L+I D    +  +L  ++W  T  F  PD
Sbjct: 56  GNACVLLALRTTRQKHSRLFFFMKHLSIADLVVAVFQVLPQLLWDITFRFYGPDLLCRLV 115

Query: 125 XXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIPTLII 184
                  ASTY+L+ +S+DR  AI  P++ L+  +  R+ ++  W    + S P + I
Sbjct: 116 KYLQVVGMFASTYLLLLMSLDRCLAICQPLRSLR-RRTDRLAVLATWLGCLVASAPQVHI 174

Query: 185 FGKRTLSNGEVQCWALWPGDSYWTP--YMTIVAFLVYFIPLTIISIMYGIVIRTIW--IK 240
           F  R +++G   CWA++     W P  Y+T +   VY +P+ +++  YG++   IW  ++
Sbjct: 175 FSLREVADGVFDCWAVFI--QPWGPKAYITWITLAVYIVPVIVLAACYGLISFKIWQNLR 232
```

```
Sbjct: 175 FSLREVADGVFDCWAVFI--QPWGPKAYITWITLAVYIVPVIVLAACYGLISFKIWQNLR 232

Query: 241 SKT------YETVISNCSDG---KLCSSYNRGLXXXXXXXXXXXXXXXXLAFICCWSPY 290
            KT           +   DG    L    +  L                 LAFI CW+P+
Sbjct: 233 LKTAAAAAAEAPEGAAAGDGGRVALARVSSVKLISKAKIRTVKMTFIIVLAFIVCWTPF 291
```

>gi|4557345|ref|NP_000045.1| arginine vasopressin receptor 2 [Homo sapiens]
gi|267256|sp|P30518|V2R_HUMAN Vasopressin V2 receptor (Renal-type arginine vaso (Antidiuretic hormone receptor) (AVPR V2)
gi|2136356|pir||I51865 vasopressin V2 receptor - human
gi|28418|emb|CAA77746.1| antidiuretic hormone receptor [Homo sapiens]
gi|398043|gb|AAA03651.1| vasopressin V2 receptor
gi|532760|gb|AAC09005.1| arginine vasopressin receptor type II, V2 antidiuretic receptor [Homo sapiens]
gi|2623240|gb|AAB86428.1| vasopressin V2 receptor [Homo sapiens]
gi|4323605|gb|AAD16444.1| vasopressin receptor type 2 [Homo sapiens]
gi|29825389|gb|AAO92298.1| arginine vasopressin receptor 2 [Homo sapiens]
gi|228760|prf||1810437A vasopressin receptor
Length = 371

```
Score = 80.1 bits (196), Expect = 6e-15
Identities = 66/283 (23%), Positives = 114/283 (40%), Gaps = 13/283 (4%)

Query: 58  VLFVFTIVGNSVVLFSTWRRKKKSR---MTFFVTQLAITDSFTGLVNILTDIIWRFTGDF 114
            ++FV   + N +VL +  RR ++      +  F+  L + D     L  +L  + W+ T  F
Sbjct: 46  IVFVAVALSNGLVLAALARRGRRGHWAPIHVFIGHLCLADLAVALFQVLPQLAWKATDRF 105

Query: 115 TAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPM---KFLQGEKQARVLIVIAW 171
              PD                AS+Y+++++++DR+ AI  PM   +   G    R ++V AW
Sbjct: 106 RGPDALCRAVKYLQMVGMYASSYMILAMTLDRHRAICRPMLAYRHGSGAHWNRPVLV-AW 164

Query: 172 SLSFLFSIPTLIIFGKRTLSNGE--VQCWALWPGDSYWTPYMTIVAFLVYFIPLTIISIM 229
            + S L S+P L IF +R +  G      CWA +         Y+T +A +V+  P    I+
Sbjct: 165 AFSLLLSLPQLFIFAQRNVEGGSGVTDCWACFAEPWGRRTYVTWIALMVFVAPTLGIAAC 224

Query: 230 YGIVIRTIW--IKSKTYETVISNCSDGKLCSSYNRGLXXXXXXXXXXXXXXXXXLAFICCW 287
              ++ R I    +     E          +  S                      + ++ CW
Sbjct: 225 QVLIFREIHASLVPGPSERPGGRRRGRRTGSPGEGAHVSAAVAKTVRMTLVIVVVYVLCW 284

Query: 288 SPYXXXXXXXXXXXXPDTQERFYASVIIQNLPALNSAINPPIY 330
            +P+              P+         V++  L +LNS  NP IY
Sbjct: 285 APF--FLVQLWAAWDPEAPLEGAPFVLLMLLASLNSCTNPWIY 325
```

>gi|447224|prf||1913493A vasopressin receptor:ISOTYPE=V2
Length = 371

```
Score = 79.7 bits (195), Expect = 7e-15
Identities = 66/283 (23%), Positives = 114/283 (40%), Gaps = 13/283 (4%)

Query: 58  VLFVFTIVGNSVVLFSTWRRKKKSR---MTFFVTQLAITDSFTGLVNILTDIIWRFTGDF 114
            ++FV   + N +VL +  RR ++      +  F+  L + D     L  +L  + W+ T  F
Sbjct: 46  IVFVAVALSNGLVLAALARRGRRGHWAPIHVFIGHLCLADLAVALFQVLPQLAWKATDRF 105
```

```
Query: 115 TAPDXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPM---KFLQGEKQARVLIVIAW 171
            PD               AS+Y+++++++DR+ AI  PM   +   G    R ++V AW
Sbjct: 106 PGPDALCRAVKYLQMVGMYASSYMILAMTLDRHRAICRPMLAYRHGSGAHWNRPVLV-AW 164

Query: 172 SLSFLFSIPTLIIFGKRTLSNGE--VQCWALWPGDSYWTPYMTIVAFLVYFIPLTIISIM 229
            + S L S+P L IF +R +  G     CWA +        Y+T +A +V+  P   I+
Sbjct: 165 AFSLLLSLPQLFIFAQRNVEGGSGVTDCWACFAEPWGRRTYVTWIALMVFVAPTLGIAAC 224

Query: 230 YGIVIRTIW--IKSKTYETVISNCSDGKLCSSYNRGLXXXXXXXXXXXXXXXXLAFICCW 287
            ++ R I   +     E          +  S                        + ++ CW
Sbjct: 225 QVLIFREIHASLVPGPSERPGGRRRGRRTGSPGEGAHVSAAVAKTVRMTLVIVVVYVLCW 284

Query: 288 SPYXXXXXXXXXXXXPDTQERFYASVIIQNLPALNSAINPPIY 330
            +P+             P+        V++  L +LNS  NP IY
Sbjct: 285 APF--FLVQLWAAWDPEAPLEGAPFVLLMLLASLNSCTNPWIY 325
```

>gi|2654031|gb|AAB87678.1| vasopressin receptor type 2 [Homo sapiens]
gi|4323607|gb|AAD16445.1| truncated vasopressin receptor type 2 [Homo sapiens]
Length = 309

Score = 79.7 bits (195), Expect = 8e-15
Identities = 51/188 (27%), Positives = 88/188 (46%), Gaps = 9/188 (4%)

```
Query: 58  VLFVFTIVGNSVVLFSTWRRKKKSR---MTFFVTQLAITDSFTGLVNILTDIIWRFTGDF 114
            ++FV   + N +VL +  RR ++      +  F+  L + D    L  +L  + W+ T  F
Sbjct: 46  IVFVAVALSNGLVLAALARRGRRGHWAPIHVFIGHLCLADLAVALFQVLPQLAWKATDRF 105

Query: 115 TAPDXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPM---KFLQGEKQARVLIVIAW 171
            PD               AS+Y+++++++DR+ AI  PM   +   G    R ++V AW
Sbjct: 106 RGPDALCRAVKYLQMVGMYASSYMILAMTLDRHRAICRPMLAYRHGSGAHWNRPVLV-AW 164

Query: 172 SLSFLFSIPTLIIFGKRTLSNGE--VQCWALWPGDSYWTPYMTIVAFLVYFIPLTIISIM 229
            + S L S+P L IF +R +  G     CWA +        Y+T +A +V+  P   I+
Sbjct: 165 AFSLLLSLPQLFIFAQRNVEGGSGVTDCWACFAEPWGRRTYVTWIALMVFVAPTLGIAAC 224

Query: 230 YGIVIRTI 237
            ++ R I
Sbjct: 225 QVLIFREI 232
```

>gi|5453666|ref|NP_006134.1| G protein-coupled receptor 19 [Homo sapiens]
gi|2495036|sp|Q15760|GP19_HUMAN Probable G protein-coupled receptor GPR19 (GPR-
gi|1323696|gb|AAB00316.1| GPR-NGA
gi|1575513|gb|AAB49751.1| putative G protein-coupled receptor; GPCR
gi|48145781|emb|CAG33113.1| GPR19 [Homo sapiens]
Length = 415

Score = 75.5 bits (184), Expect = 1e-13
Identities = 55/183 (30%), Positives = 86/183 (46%), Gaps = 7/183 (3%)

```
Query: 58  VLFVFTIVGNSVVLFSTWR-RKKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTA 116
            +L++F+I GNS+V     R R+ +S   +FV  +A  D    + +    ++   TG +T
Sbjct: 74  ILWLFSIFGNSLVCLVIHRSRRTQSTTNYFVVSMACADLLISVASTPFVLLQFTTGRWTL 133

Query: 117 PDXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFL 176
```

```
                                YVL+S+ IDR++ IVYP+ F    ++A+ +I  +W
Sbjct: 134 GSATCKVVRYFQYLTPGVQIYVLLSICIDRFYTIVYPLSFKVSREKAKKMIAASWIFDAG 193

Query: 177 FSIPTLIIFGKRTLSNGEVQCWALWPGDSYWTPYMTIVAFLVYF-IPLTIISIMYGIVIR 235
           F  P L  +G    SN +  C    P     T Y T++ FLV F IP  +I + Y  VI+
Sbjct: 194 FVTPVLFFYG----SNWDSHCNYFLPSSWEGTAY-TVIHFLVGFVIPSVLIILFYQKVIK 248

Query: 236 TIW 238
            IW
Sbjct: 249 YIW 251
```

>gi|7513259|pir||S74237 probable G protein-coupled receptor - human
Length = 415

```
 Score = 75.5 bits (184), Expect = 1e-13
 Identities = 55/183 (30%), Positives = 86/183 (46%), Gaps = 7/183 (3%)

Query: 58  VLFVFTIVGNSVVLFSTWR-RKKKSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTA 116
           +L++F+I GNS+V     R R+ +S   +FV  +A  D    + +    ++   TG +T
Sbjct: 74  ILWLFSIFGNSLVCLVIHRSRRTQSTTNYFVVSMACADLLISVASTPFVLLQFTTGRWTL 133

Query: 117 PDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFL 176
                               YVL+S+ IDR++ IVYP+ F    ++A+ +I  +W
Sbjct: 134 GSATCKVVRYFQYLTPGVQIYVLLSICIDRFYTIVYPLSFKVSREKAKKMIAASWIFDAG 193

Query: 177 FSIPTLIIFGKRTLSNGEVQCWALWPGDSYWTPYMTIVAFLVYF-IPLTIISIMYGIVIR 235
           F  P L  +G    SN +  C    P     T Y T++ FLV F IP  +I + Y  VI+
Sbjct: 194 FVTPVLFFYG----SNWDSHCNYFLPSSWEGTAY-TVIHFLVGFVIPSVLIILFYQKVIK 248

Query: 236 TIW 238
            IW
Sbjct: 249 YIW 251
```

>gi|246909|gb|AAB21706.1| neurokinin-3 receptor; NK-3 receptor [Homo sapiens]
Length = 465

```
 Score = 72.4 bits (176), Expect = 1e-12
 Identities = 69/289 (23%), Positives = 111/289 (38%), Gaps = 21/289 (7%)

Query: 54  ITLWVL-----FVFTIVGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSFTGLVNILTDII 107
           I LW L         ++GN +V++     K+    +T +F+  LA +D+     N L + I
Sbjct: 85  IALWSLAYGVVVAVAVLGNLIVIWIILAHKRMRTVTNYFLVNLAFSDASMAAFNTLVNFI 144

Query: 108 WRFTGDFTAPDXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLI 167
           +    ++                    AS Y + ++++DRY AI+ P+K        +++I
Sbjct: 145 YALHSEWYFGANYCRFQNFFPITAVFASIYSMTAIAVDRYMAIIDPLKPRLSATATKIVI 204

Query: 168 VIAWSLSFLFSIPTLIIFGKRTLSNGEVQCWALWP-GDSYWTPYMTIVAFLVYFIPLTII 226
              W L+FL + P   ++ K  +  G   C+  WP G      Y  IV  LVY  PL I+
Sbjct: 205 GSIWILAFLLAFPQ-CLYSKTKVMPGRTLCFVQWPEGPKQHFTYHIIVIILVYCFPLLIM 263

Query: 227 SIMYGIVIRTIWIKSKTYETVISNCSDGKLCSSYNRGLXXXXXXXXXXXXXXXLAFICC 286
           I Y IV  T+W               G  C  Y+  L                + F  C
Sbjct: 264 GITYTIVGITLW----------GGEIPGDTCDKYHEQL--KAKRKVVKMMIIVVMTFAIC 311
```

```
Query: 287 WSPYXXXXXXXXXXXXPDTQERFYASVIIQNLPALNSAI-NPPIYCVFS 334
          W PY             +  +      +     A++S + NP IYC  +
Sbjct: 312 WLPYHIYFILTAIYQQLNRWKYIQQVYLASFWLAMSSTMYNPIIYCCLN 360
```

>gi|7669548|ref|NP_001050.1| tachykinin receptor 3; NK-3 receptor; neurokinin sapiens]
gi|128364|sp|P29371|NK3R_HUMAN Neuromedin K receptor (NKR) (Neurokinin B recept (NK-3R) (Tachykinin receptor 3)
gi|88252|pir||JQ1517 neurokinin 3 receptor - human
gi|189224|gb|AAA36366.1| neurokinin-3 receptor
gi|38565063|gb|AAR23926.1| tachykinin receptor 3 [Homo sapiens]
Length = 465

Score = 72.4 bits (176), Expect = 1e-12
Identities = 69/289 (23%), Positives = 111/289 (38%), Gaps = 21/289 (7%)

```
Query: 54  ITLWVL-----FVFTIVGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSFTGLVNILTDII 107
           I LW L          ++GN +V++      K+    +T +F+  LA +D+      N L + I
Sbjct: 85  IALWSLAYGVVVAVAVLGNLIVIWIILAHKRMRTVTNYFLVNLAFSDASMAAFNTLVNFI 144

Query: 108 WRFTGDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLI 167
           +    ++                    AS Y + ++++DRY AI+ P+K         +++I
Sbjct: 145 YALHSEWYFGANYCRFQNFFPITAVFASIYSMTAIAVDRYMAIIDPLKPRLSATATKIVI 204

Query: 168 VIAWSLSFLFSIPTLIIFGKRTLSNGEVQCWALWP-GDSYWTPYMTIVAFLVYFIPLTII 226
              W L+FL + P   ++ K  +  G   C+  WP G      Y  IV  LVY  PL I+
Sbjct: 205 GSIWILAFLLAFPQ-CLYSKTKVMPGRTLCFVQWPEGPKQHFTYHIIVIILVYCFPLLIM 263

Query: 227 SIMYGIVIRTIWIKSKTYETVISNCSDGKLCSSYNRGLXXXXXXXXXXXXXXXLAFICC 286
            I Y IV  T+W               G  C  Y+  L                + F  C
Sbjct: 264 GITYTIVGITLW----------GGEIPGDTCDKYHEQL--KAKRKVVKMMIIVVMTFAIC 311

Query: 287 WSPYXXXXXXXXXXXXPDTQERFYASVIIQNLPALNSAI-NPPIYCVFS 334
           W PY             +  +      +     A++S + NP IYC  +
Sbjct: 312 WLPYHIYFILTAIYQQLNRWKYIQQVYLASFWLAMSSTMYNPIIYCCLN 360
```

>gi|825695|emb|CAA46291.1| neuromedin K receptor [Homo sapiens]
Length = 465

Score = 72.4 bits (176), Expect = 1e-12
Identities = 69/289 (23%), Positives = 111/289 (38%), Gaps = 21/289 (7%)

```
Query: 54  ITLWVL-----FVFTIVGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSFTGLVNILTDII 107
           I LW L          ++GN +V++      K+    +T +F+  LA +D+      N L + I
Sbjct: 85  IALWSLAYGVVVAVAVLGNLIVIWIILAHKRMRTVTNYFLVNLAFSDASMAAFNTLVNFI 144

Query: 108 WRFTGDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLI 167
           +    ++                    AS Y + ++++DRY AI+ P+K         +++I
Sbjct: 145 YALHSEWYFGANYCRFQNFFPITAVFASIYSMTAIAVDRYMAIIDPLKPRLSATATKIVI 204

Query: 168 VIAWSLSFLFSIPTLIIFGKRTLSNGEVQCWALWP-GDSYWTPYMTIVAFLVYFIPLTII 226
              W L+FL + P   ++ K  +  G   C+  WP G      Y  IV  LVY  PL I+
Sbjct: 205 GSIWILAFLLAFPQ-CLYSKTKVMPGRTLCFVQWPEGPKQHFTYHIIVIILVYCFPLLIM 263
```

```
Query: 227 SIMYGIVIRTIWIKSKTYETVISNCSDGKLCSSYNRGLXXXXXXXXXXXXXXXXLAFICC 286
           I Y IV  T+W               G  C  Y+  L                 + F  C
Sbjct: 264 GITYTIVGITLW----------GGEIPGDTCDKYHEQL--KAKRKVVKMMIIVVMTFAIC 311

Query: 287 WSPYXXXXXXXXXXXXPDTQERFYASVIIQNLPALNSAI-NPPIYCVFS 334
           W PY             +  +      +     A++S + NP IYC  +
Sbjct: 312 WLPYHIYFILTAIYQQLNRWKYIQQVYLASFWLAMSSTMYNPIIYCCLN 360
```

>gi|21928727|dbj|BAC05950.1| seven transmembrane helix receptor [Homo sapiens]
Length = 441

```
 Score = 71.2 bits (173), Expect = 3e-12
 Identities = 43/197 (21%), Positives = 87/197 (44%), Gaps = 12/197 (6%)

Query: 53  LITLWVLFVFTIVGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSFTGLVNILTDIIWRFT 111
           ++   ++F+  +VGN++V F    + +     +T  F+  LA++D   G+  + T ++
Sbjct: 58  IVAYALIFLLCMVGNTLVCFIVLKNRHMHTVTNMFILNLAVSDLLVGIFCMPTTLVDNLI 117

Query: 112 GDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAW 171
             +   +                AS + LV+++++R+  IV+P +     ++A V I + W
Sbjct: 118 TGWPFDNATCKMSGLVQGMSVSASVFTLVAIAVERFRCIVHPFREKLTLRKALVTIAVIW 177

Query: 172 SLSFLFSIPTLI-----------IFGKRTLSNGEVQCWALWPGDSYWTPYMTIVAFLVYF 220
           +L+ L    P+ +           +   R  S     CW  WP       Y T++   +Y
Sbjct: 178 ALALLIMCPSAVTLTVTREEHHFMVDARNRSYPLYSCWEAWPEKGMRRVYTTVLFSHIYL 237

Query: 221 IPLTIISIMYGIVIRTI 237
            PL +I +MY  + R +
Sbjct: 238 APLALIVVMYARIARKL 254
```

>gi|15281400|gb|AAK94199.1| neuropeptide NPVF receptor [Homo sapiens]
Length = 430

```
 Score = 70.9 bits (172), Expect = 3e-12
 Identities = 43/197 (21%), Positives = 87/197 (44%), Gaps = 12/197 (6%)

Query: 53  LITLWVLFVFTIVGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSFTGLVNILTDIIWRFT 111
           ++   ++F+  +VGN++V F    + +     +T  F+  LA++D   G+  + T ++
Sbjct: 47  IVAYALIFLLCMVGNTLVCFIVLKNRHMHTVTNMFILNLAVSDLLVGIFCMPTTLVDNLI 106

Query: 112 GDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAW 171
             +   +                AS + LV+++++R+  IV+P +     ++A V I + W
Sbjct: 107 TGWPFDNATCKMSGLVQGMSVSASVFTLVAIAVERFRCIVHPFREKLTLRKALVTIAVIW 166

Query: 172 SLSFLFSIPTLI-----------IFGKRTLSNGEVQCWALWPGDSYWTPYMTIVAFLVYF 220
           +L+ L    P+ +           +   R  S     CW  WP       Y T++   +Y
Sbjct: 167 ALALLIMCPSAVTLTVTREEHHFMVDARNRSYPLYSCWEAWPEKGMRRVYTTVLFSHIYL 226

Query: 221 IPLTIISIMYGIVIRTI 237
            PL +I +MY  + R +
Sbjct: 227 APLALIVVMYARIARKL 243
```

>gi|11545887|ref|NP_071429.1| G protein-coupled receptor 147; neuropeptide FF

peptide receptor [Homo sapiens]
gi|13878599|sp|Q9GZQ6|NFF1_HUMAN Neuropeptide FF receptor 1 (RFamide-related pe
OT7T022)
gi|11125702|dbj|BAB17677.1| RFamide-related peptide receptor [Homo sapiens]
gi|11907913|gb|AAG41397.1| neuropeptide FF receptor 1 [Homo sapiens]
Length = 430

Score = 70.9 bits (172), Expect = 3e-12
Identities = 43/197 (21%), Positives = 87/197 (44%), Gaps = 12/197 (6%)

```
Query: 53  LITLWVLFVFTIVGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSFTGLVNILTDIIWRFT 111
           ++   ++F+  +VGN++V F   + +    +T  F+  LA++D   G+  + T ++
Sbjct: 47  IVAYALIFLLCMVGNTLVCFIVLKNRHMHTVTNMFILNLAVSDLLVGIFCMPTTLVDNLI 106

Query: 112 GDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAW 171
             +   +               AS + LV+++++R+  IV+P +     ++A V I + W
Sbjct: 107 TGWPFDNATCKMSGLVQGMSVSASVFTLVAIAVERFRCIVHPFREKLTLRKALVTIAVIW 166

Query: 172 SLSFLFSIPTLI-----------IFGKRTLSNGEVQCWALWPGDSYWTPYMTIVAFLVYF 220
           +L+ L   P+ +           +   R  S     CW  WP       Y T++   +Y
Sbjct: 167 ALALLIMCPSAVTLTVTREEHHFMVDARNRSYPLYSCWEAWPEKGMRRVYTTVLFSHIYL 226

Query: 221 IPLTIISIMYGIVIRTI 237
            PL +I +MY  + R +
Sbjct: 227 APLALIVVMYARIARKL 243
```

>gi|13878604|sp|Q9Y5X5|NFF2_HUMAN Neuropeptide FF receptor 2 (Neuropeptide G p
receptor) (G protein-coupled receptor 74)
(G-protein-coupled receptor HLWAR77)
Length = 522

Score = 69.3 bits (168), Expect = 1e-11
Identities = 53/198 (26%), Positives = 89/198 (44%), Gaps = 25/198 (12%)

```
Query: 53  LITLWVLFVFTIVGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSFTGL----VNILTDII 107
           +I+ +++F   ++GN+VV F   R K    +T  F+  LAI+D   G+    + +L +II
Sbjct: 151 IISYFLIFFLCMMGNTVVCFIVMRNKHMHTVTNLFILNLAISDLLVGIFCMPITLLDNII 210

Query: 108 --WRFTGDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARV 165
             W F       +                AS + LV++++DR+  +VYP K     K A V
Sbjct: 211 AGWPFG------NTMCKISGLVQGISVAASVFTLVAIAVDRFQCVVYPFKPKLTIKTAFV 264

Query: 166 LIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQ-----------CWALWPGDSYWTPYMTI 213
           +I+I W L+     P+ ++   +      V+            C   WP       Y T+
Sbjct: 265 IIMIIWVLAITIMSPSAVMLHVQEEKYYRVRLNSQNKTSPVYWCREDWPNQEMRKIYTTV 324

Query: 214 VAFLVYFIPLTIISIMYG 231
           +   +Y  PL++I IMYG
Sbjct: 325 LFANIYLAPLSLIVIMYG 342
```

>gi|16604258|ref|NP_444264.1| G protein-coupled receptor 74; neuropeptide G p
receptor; neuropeptide FF 2 [Homo sapiens]
gi|9309469|gb|AAF87078.1| G-protein coupled receptor HLWAR77 [Homo sapiens]
gi|11907915|gb|AAG41398.1| neuropeptide FF receptor 2 [Homo sapiens]

gi|15281396|gb|AAK94197.1| neuropeptide NPFF receptor [Homo sapiens]
Length = 420

Score = 69.3 bits (168), Expect = 1e-11
Identities = 53/198 (26%), Positives = 89/198 (44%), Gaps = 25/198 (12%)

```
Query: 53  LITLWVLFVFTIVGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSFTGL----VNILTDII 107
           +I+ +++F   ++GN+VV F   R K    +T  F+  LAI+D   G+    + +L +II
Sbjct: 49  IISYFLIFFLCMMGNTVVCFIVMRNKHMHTVTNLFILNLAISDLLVGIFCMPITLLDNII 108

Query: 108 --WRFTGDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARV 165
             W F       +                AS + LV++++DR+  +VYP K     K A V
Sbjct: 109 AGWPFG------NTMCKISGLVQGISVAASVFTLVAIAVDRFQCVVYPFKPKLTIKTAFV 162

Query: 166 LIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQ----------CWALWPGDSYWTPYMTI 213
           +I+I W L+     P+ ++   +      V+            C   WP       Y T+
Sbjct: 163 IIMIIWVLAITIMSPSAVMLHVQEEKYYRVRLNSQNKTSPVYWCREDWPNQEMRKIYTTV 222

Query: 214 VAFLVYFIPLTIISIMYG 231
           +   +Y  PL++I IMYG
Sbjct: 223 LFANIYLAPLSLIVIMYG 240
```

>gi|4758820|ref|NP_004876.1| G protein-coupled receptor 74; neuropeptide G pr
receptor; neuropeptide FF 2 [Homo sapiens]
gi|4530469|gb|AAD22047.1| G-protein-coupled receptor [Homo sapiens]
Length = 522

Score = 69.3 bits (168), Expect = 1e-11
Identities = 53/198 (26%), Positives = 89/198 (44%), Gaps = 25/198 (12%)

```
Query: 53  LITLWVLFVFTIVGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSFTGL----VNILTDII 107
           +I+ +++F   ++GN+VV F   R K    +T  F+  LAI+D   G+    + +L +II
Sbjct: 151 IISYFLIFFLCMMGNTVVCFIVMRNKHMHTVTNLFILNLAISDLLVGIFCMPITLLDNII 210

Query: 108 --WRFTGDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARV 165
             W F       +                AS + LV++++DR+  +VYP K     K A V
Sbjct: 211 AGWPFG------NTMCKISGLVQGISVAASVFTLVAIAVDRFQCVVYPFKPKLTIKTAFV 264

Query: 166 LIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQ----------CWALWPGDSYWTPYMTI 213
           +I+I W L+     P+ ++   +      V+            C   WP       Y T+
Sbjct: 265 IIMIIWVLAITIMSPSAVMLHVQEEKYYRVRLNSQNKTSPVYWCREDWPNQEMRKIYTTV 324

Query: 214 VAFLVYFIPLTIISIMYG 231
           +   +Y  PL++I IMYG
Sbjct: 325 LFANIYLAPLSLIVIMYG 342
```

>gi|14279165|gb|AAK58513.1| G-protein-coupled receptor 74 [Homo sapiens]
Length = 408

Score = 68.6 bits (166), Expect = 2e-11
Identities = 53/198 (26%), Positives = 89/198 (44%), Gaps = 25/198 (12%)

```
Query: 53  LITLWVLFVFTIVGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSFTGL----VNILTDII 107
           +I+ +++F   ++GN+VV F   R K    +T  F+  LAI+D   G+    + +L +II
Sbjct: 52  IISYFLIFFLCMMGNTVVCFIVMRNKHMHTVTNLFILNLAISDLLVGIFCMPITLLDNII 111
```

```
Query: 108 --WRFTGDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARV 165
             W F       +                AS + LV++++DR+  +VYP K     K A V
Sbjct: 112 AGWPFG------NTMCKISGLVQGISVAASVFTLVAIAVDRFQCVVYPFKPKLTIKTAFV 165

Query: 166 LIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQ------------CWALWPGDSYWTPYMTI 213
           +I+I W L+     P+ ++   +      V+             C   WP       Y T+
Sbjct: 166 IIMIIWVLAITIMSPSAVMLHVQEEKYYRVRLNSQNKTSPVYWCREDWPNQEMRKIYTTV 225

Query: 214 VAFLVYFIPLTIISIMYG 231
           +   +Y  PL++I IMYG
Sbjct: 226 LFANIYLAPLSLIVIMYG 243
```

>gi|4505513|ref|NP_000904.1| opiate receptor-like 1; rat LC132 receptor-like; opioid receptor; nociceptin receptor; orphanin FQ receptor [Homo sapiens]

gi|33286424|ref|NP_872588.1| opiate receptor-like 1; rat LC132 receptor-like; k opioid receptor; nociceptin receptor; orphanin FQ receptor [Homo sapiens]

gi|730230|sp|P41146|OPRX_HUMAN Nociceptin receptor (Orphanin FQ receptor) (Kapp receptor) (KOR-3)

gi|631318|pir||S43087 orphan opioid receptor ORL1 - human

gi|471317|emb|CAA54386.1| ORL1 [Homo sapiens]

gi|1144297|gb|AAA84913.1| orphan opioid receptor

gi|13022243|gb|AAK11714.1| nociceptin receptor [Homo sapiens]

gi|23468341|gb|AAH38433.1| OPRL1 protein [Homo sapiens]

gi|30385528|gb|AAP23195.1| opiate receptor-like 1 [Homo sapiens]

gi|49457075|emb|CAG46858.1| OPRL1 [Homo sapiens]

Length = 370

Score = 65.5 bits (158), Expect = 2e-10
Identities = 49/181 (27%), Positives = 88/181 (48%), Gaps = 11/181 (6%)

```
Query: 64  IVGNSVVLFSTWRRKK-KSRMTFFVTQLAITDSFTGLVNIL--TDIIWRFTGDFTAPDXX 120
           ++GN +V++   R  K K+    ++  LA+ D+   L      TDI+  F   +   +
Sbjct: 66  LLGNCLVMYVILRHTKMKTATNIYIFNLALADTLVLLTLPFQGTDILLGF---WPFGNAL 122

Query: 121 XXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFL--QGEKQARVLIVIAWSLSFLFS 178
                       ST+ L ++S+DRY AI +P++ L  +    +A+ + V  W+L+ +
Sbjct: 123 CKTVIAIDYYNMFTSTFTLTAMSVDRYVAICHPIRALDVRTSSKAQAVNVAIWALASVVG 182

Query: 179 IPTLIIFGKRTLSNGEVQCWALWPG-DSYWTPYMTIVAFLVYFI-PLTIISIMYGIVIRT 236
           +P  I+ G   + + E++C    P    YW P   I  FL  FI P+ +IS+ Y ++IR
Sbjct: 183 VPVAIM-GSAQVEDEEIECLVEIPTPQDYWGPVFAICIFLFSFIVPVLVISVCYSLMIRR 241

Query: 237 I 237
           +
Sbjct: 242 L 242
```

>gi|542922|pir||A44081 kappa-type opioid receptor - human

gi|189392|gb|AAA36395.1| putative opioid receptor

Length = 440

```
 Score = 65.1 bits (157), Expect = 2e-10
 Identities = 68/273 (24%), Positives = 105/273 (38%), Gaps = 16/273 (5%)

Query: 65  VGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSFTGLVNILTDIIWRFTGDFTAPDXXXXX 123
           +GN VV++     K+   +T  F+  LA  D+    +N L + I+   G++
Sbjct: 76  LGNLVVIWIVLAHKRMRTVTNSFLVNLAFADAAMAALNALVNFIYALHGEWYFGANYCRF 135

Query: 124 XXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFLFSIPTLI 183
                   AS Y + ++++DRY AI+ P+K        R++I   W L+FL + P
Sbjct: 136 QNFFPITAVFASIYSMTAIAVDRYMAIIDPLKPRLSATATRIVIGSIWILAFLLAFPQ-C 194

Query: 184 IFGKRTLSNGEVQCWALWP-GDSYWTPYMTIVAFLVYFIPLTIISIMYGIVIRTIWIKSK 242
           ++ K  +  G   C+  WP G      Y  IV  LVY  PL I+ I Y IV  T+W
Sbjct: 195 LYSKIKVMPGRTLCYVQWPEGSRQHFTYHMIVIVLVYCFPLLIMGITYTIVGITLW---- 250

Query: 243 TYETVISNCSDGKLCSSYNRGLXXXXXXXXXXXXXXXXLAFICCWSPYXXXXXXXXXXXX 302
                    G  C  Y   L                + F  CW PY
Sbjct: 251 ------GGEIPGDTCDKYQEQL--KAKRKVVKMMIIVVVTFAICWLPYHIYFILTAIYQQ 302

Query: 303 PDTQERFYASVIIQNLPALNSAI-NPPIYCVFS 334
            +  +      +     A++S + NP IYC  +
Sbjct: 303 LNRWKYIQQVYLASFWLAMSSTMYNPIIYCCLN 335
```

>gi|725266|gb|AAA96251.1| opioid receptor-like protein
gi|1585750|prf||2201468A opioid orphan receptor
Length = 333

```
 Score = 64.7 bits (156), Expect = 3e-10
 Identities = 49/181 (27%), Positives = 88/181 (48%), Gaps = 11/181 (6%)

Query: 64  IVGNSVVLFSTWRRKK-KSRMTFFVTQLAITDSFTGLVNIL--TDIIWRFTGDFTAPDXX 120
           ++GN +V++    R  K K+     ++  LA+ D+    L      TDI+  F    +
Sbjct: 66  LLGNCLVMYVILRHTKMKTATNIYIFNLALADTLVLLTLPFQGTDILLGF---WPFGNAL 122

Query: 121 XXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFL--QGEKQARVLIVIAWSLSFLFS 178
                       ST+ L ++S+DRY AI +P++ L  +    +A+ + V  W+L+ +
Sbjct: 123 CKTVIAIDYYNMFTSTFTLTAMSVDRYVAICHPIRALDVRTSSKAQAVNVAIWALASVVG 182

Query: 179 IPTLIIFGKRTLSNGEVQCWALWPG-DSYWTPYMTIVAFLVYFI-PLTIISIMYGIVIRT 236
           +P  I+ G    + + E++C    P    YW P   I   FL  FI P+ +IS+ Y ++IR
Sbjct: 183 VPVAIM-GSAQVEDEEIECLVEIPTPQDYWGPVFAICIFLFSFIVPVLVISVCYSLMIRR 241

Query: 237 I 237
           +
Sbjct: 242 L 242
```

>gi|49457117|emb|CAG46879.1| OPRL1 [Homo sapiens]
Length = 370

```
 Score = 64.3 bits (155), Expect = 3e-10
 Identities = 49/181 (27%), Positives = 88/181 (48%), Gaps = 11/181 (6%)

Query: 64  IVGNSVVLFSTWRRKK-KSRMTFFVTQLAITDSFTGLVNIL--TDIIWRFTGDFTAPDXX 120
           ++GN +V++    R  K K+     ++  LA+ D+    L      TDI+  F    +
Sbjct: 66  LLGNCLVMYVILRHTKMKTATNIYIFNLALADTLVLLTLPFQGTDILLGF---WPFGNAL 122
```

```
Query: 121 XXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFL--QGEKQARVLIVIAWSLSFLFS 178
                        ST+ L ++S+DRY AI +P++ L  +   +A+ + V  W+L+ +
Sbjct: 123 CKTVIAIDYYNMFTSTFTLTAMSVDRYVAICHPIRALDVRTSSKAQAVNVAIWALASVVG 182

Query: 179 IPTLIIFGKRTLSNGEVQCWALWPG-DSYWTPYMTIVAFLVYFI-PLTIISIMYGIVIRT 236
           +P  I+ G   + + E++C    P    YW P   I  FL  FI P+ +IS+ Y ++IR
Sbjct: 183 VPVAIM-GSAQVEDEEIECPVEIPTPQDYWGPVFAICIFLFSFIVPVLVISVCYSLMIRR 241

Query: 237 I 237
           +
Sbjct: 242 L 242
```

>gi|4557859|ref|NP_001041.1| somatostatin receptor 2 [Homo sapiens]
gi|401126|sp|P30874|SSR2_HUMAN Somatostatin receptor type 2 (SS2R) (SRIF-1)
gi|284408|pir||B41795 somatostatin receptor 2 - human
gi|307436|gb|AAA58248.1| somatostatin receptor isoform 2
gi|7229403|gb|AAF42809.1| somatostatin receptor 2A [Homo sapiens]
gi|18043109|gb|AAH19610.1| Somatostatin receptor 2 [Homo sapiens]
gi|21929083|dbj|BAC06126.1| seven transmembrane helix receptor [Homo sapiens]
gi|29824924|gb|AAO92064.1| somatostatin receptor 2 [Homo sapiens]
Length = 369

Score = 63.5 bits (153), Expect = 6e-10
Identities = 52/202 (25%), Positives = 92/202 (45%), Gaps = 18/202 (8%)

```
Query: 45  YYSFKTEQLITL--WVLFVFTIVGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSF--TGL 99
           YY   +  ++T   +V+ +  + GN++V++   R  K   +T  ++  LAI D     GL
Sbjct: 37  YYDLTSNAVLTFIYFVVCIIGLCGNTLVIYVILRYAKMKTITNIYILNLAIADELFMLGL 96

Query: 100 VNILTDII---WRFTGDFTAPDXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKF 156
             +   +    W F                          S + L  +SIDRY A+V+P+K
Sbjct: 97  PFLAMQVALVHWPFG------KAICRVVMTVDGINQFTSIFCLTVMSIDRYLAVVHPIKS 150

Query: 157 LQGEK--QARVLIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQCWALWPGDS--YWTPYMT 212
            +  +   A+++ +  W +S L  +P +I  G R+   G   C   WPG+S  ++T ++
Sbjct: 151 AKWRRPRTAKMITMAVWGVSLLVILPIMIYAGLRSNQWGRSSCTINWPGESGAWYTGFII 210

Query: 213 IVAFLVYFIPLTIISIMYGIVI 234
              L + +PLTII + Y  +I
Sbjct: 211 YTFILGFLVPLTIICLCYLFII 232
```

>gi|7229404|gb|AAF42810.1| somatostatin receptor 2B [Homo sapiens]
Length = 356

Score = 63.5 bits (153), Expect = 6e-10
Identities = 52/202 (25%), Positives = 92/202 (45%), Gaps = 18/202 (8%)

```
Query: 45  YYSFKTEQLITL--WVLFVFTIVGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSF--TGL 99
           YY   +  ++T   +V+ +  + GN++V++   R  K   +T  ++  LAI D     GL
Sbjct: 37  YYDLTSNAVLTFIYFVVCIIGLCGNTLVIYVILRYAKMKTITNIYILNLAIADELFMLGL 96

Query: 100 VNILTDII---WRFTGDFTAPDXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKF 156
```

```
            +   +    W F                          S + L  +SIDRY A+V+P+K
Sbjct: 97   PFLAMQVALVHWPFG------KAICRVVMTVDGINQFTSIFCLTVMSIDRYLAVVHPIKS 150

Query: 157 LQGEK--QARVLIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQCWALWPGDS--YWTPYMT 212
            +  +   A+++ +  W +S L  +P +I  G R+   G   C   WPG+S  ++T ++
Sbjct: 151 AKWRRPRTAKMITMAVWGVSLLVILPIMIYAGLRSNQWGRSSCTINWPGESGAWYTGFII 210

Query: 213 IVAFLVYFIPLTIISIMYGIVI 234
               L + +PLTII + Y  +I
Sbjct: 211 YTFILGFLVPLTIICLCYLFII 232
```

>gi|4557639|ref|NP_001517.1| L orexin receptor 2 [Homo sapiens]
gi|6225810|sp|O43614|OX2R_HUMAN L Orexin receptor type 2 (Ox2r) (Hypocretin recep
gi|2897128|gb|AAC39602.1| L orexin receptor-2; OX2R; G protein-coupled receptor [
gi|11055244|gb|AAG28021.1| hypocretin receptor-2 [Homo sapiens]
gi|17978555|gb|AAL47215.1| L hypocretin receptor 2; orexin receptor 2 [Homo sapie
Length = 444

Score = 63.2 bits (152), Expect = 7e-10
Identities = 43/191 (22%), Positives = 80/191 (41%), Gaps = 8/191 (4%)

```
Query: 58  VLFVFTIVGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSFTGLVNILTDIIWRFTGDFTA 116
           ++FV  ++GN +V  + W+       +T +F+  L++ D    +  +   ++   T  +
Sbjct: 63  IVFVVALIGNVLVCVAVWKNHHMRTVTNYFIVNLSLADVLVTITCLPATLVVDITETWFF 122

Query: 117 PDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAWSLSFL 176
                          S  L  +++DR++AI +P+ F    K+AR  IVI W +S +
Sbjct: 123 GQSLCKVIPYLQTVSVSVSVLTLSCIALDRWYAICHPLMFKSTAKRARNSIVIIWIVSCI 182

Query: 177 FSIPTLIIFGKRTLSNGEVQ-------CWALWPGDSYWTPYMTIVAFLVYFIPLTIISIM 229
             IP  I+    T+  G          C   W G+ Y   Y      + Y  PL ++ +
Sbjct: 183 IMIPQAIVMECSTVFPGLANKTTLFTVCDERWGGEIYPKMYHICFFLVTYMAPLCLMVLA 242

Query: 230 YGIVIRTIWIK 240
           Y  + R +W +
Sbjct: 243 YLQIFRKLWCR 253
```

>gi|14550544|gb|AAH09522.1| L Unknown (protein for IMAGE:3354783) [Homo sapiens]
Length = 346

Score = 62.8 bits (151), Expect = 9e-10
Identities = 52/202 (25%), Positives = 92/202 (45%), Gaps = 18/202 (8%)

```
Query: 45  YYSFKTEQLITL--WVLFVFTIVGNSVVLFSTWRRKKKSRMT-FFVTQLAITDSF--TGL 99
           YY   +  ++T   +V+ +  + GN++V+   R  K   +T  ++  LAI D     GL
Sbjct: 14  YYDLTSNAVLTFIYFVVCIIGLCGNTLVIYVILRYAKMKTITNIYILNLAIADELFMLGL 73

Query: 100 VNILTDII---WRFTGDFTAPDXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKF 156
             +   +    W F                          S + L  +SIDRY A+V+P+K
Sbjct: 74  PFLAMQVALVHWPFG------KAICRVVMTVDGINQFTSIFCLTVMSIDRYLAVVHPIKS 127

Query: 157 LQGEK--QARVLIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQCWALWPGDS--YWTPYMT 212
            +  +   A+++ +  W +S L  +P +I  G R+   G   C   WPG+S  ++T ++
Sbjct: 128 AKWRRPRTAKMITMAVWGVSLLVILPIMIYAGLRSNQWGRSSCTINWPGESGAWYTGFII 187
```

```
Query: 213 IVAFLVYFIPLTIISIMYGIVI 234
              L + +PLTII + Y  +I
Sbjct: 188 YTFILGFLVPLTIICLCYLFII 209
```

>gi|28380053|sp|Q96P88|GRR2_HUMAN Gonadotropin-releasing hormone II receptor (GnRH-II-R)
Length = 379

Score = 62.8 bits (151), Expect = 1e-09
Identities = 79/331 (23%), Positives = 129/331 (38%), Gaps = 21/331 (6%)

```
Query: 38  GKEWGSFYYSFKTEQLITLWVLFVFTIVGNSVVLFSTWRRK----KKSRMTFFVTQLAIT 93
           G E  +F  + K    +T+ VLFV +  GN  VL+S  RR+    + S +      LA
Sbjct: 27  GSELPTFSAAAKVRVGVTI-VLFVSSAGGNLAVLWSVTRREPSQLRPSPVRRLFIHLAAA 85

Query: 94  DSFTGLVNILTDIIWRFTGDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYP 153
           D     V +  D  W  T  + A D                ++ ++ V + +DR  A++ P
Sbjct: 86  DLLVTFVVMPLDATWNITVQWLAVDIACRTLMFLKLMATYSAAFLPVVIGLDRQAAVLNP 145

Query: 154 MKFLQGEKQARVLIVIAWSLSFLFSIPTLIIFGKRTLSNGEV---QCWALWPGDSYW--T 208
           +    G    R L+  AW LSFL + P L +F     + G V   QC        + W  T
Sbjct: 146 LGSRSG---VRKLLGAAWGLSFLLAFPQLFLFHTVHXA-GPVPFTQCVTKGSFKAQWQET 201

Query: 209 PYMTIVAFLVYFIPLTIISIMYGIVIRTIWIKSKTYETVISNCSDGKLCSSYNRGLXXXX 268
            Y       ++ +PLT ++I Y  ++ ++   S+      S+   G+     +
Sbjct: 202 TYNLFTFCCLFLLPLTAMAICYSRIVLSV---SRPQTRKGSHAPAGEFALPRSFDNCPRV 258

Query: 269 XXXXXXXXXXXXLAFICCWSPYXXXXXXXXXXXXPDTQERFYASVIIQNLPALNSAINPP 328
                    L FI CW+PY               T+     S I+  L  LN+ ++P
Sbjct: 259 RLRALRLALLILLTFILCWTPYYLLGMWYWFSPTMLTEVPPSLSHILFLLGLLNAPLDPL 318

Query: 329 IYCVFSSSISFPCREQRSQDSRMTFRERTER 359
           +Y  F    +  CR    + S  + +E + R
Sbjct: 319 LYGAF----TLGCRRGHQELSIDSSKEGSGR 345
```

>gi|18000079|gb|AAL54890.1| KOR-3D [Homo sapiens]
Length = 365

Score = 62.4 bits (150), Expect = 1e-09
Identities = 48/180 (26%), Positives = 87/180 (48%), Gaps = 14/180 (7%)

```
Query: 64  IVGNSVVLFSTWRRKKKSRMTFFVTQLAITDSFTGLVNIL--TDIIWRFTGDFTAPDXXX 121
           ++GN +V+ +    K K+    ++  LA+ D+   L      TDI+  F   +   +
Sbjct: 66  LLGNCLVMHT----KMKTATNIYIFNLALADTLVLLTLPFQGTDILLGF---WPFGNALC 118

Query: 122 XXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFL--QGEKQARVLIVIAWSLSFLFSI 179
                     ST+ L ++S+DRY AI +P++ L   +   +A+ + V  W+L+ +  +
Sbjct: 119 KTVIAIDYYNMFTSTFTLTAMSVDRYVAICHPIRALDVRTSSKAQAVNVAIWALASVVGV 178

Query: 180 PTLIIFGKRTLSNGEVQCWALWPG-DSYWTPYMTIVAFLVYFI-PLTIISIMYGIVIRTI 237
           P  I+ G   + + E++C    P    YW P   I  FL  FI P+ +IS+ Y ++IR +
Sbjct: 179 PVAIM-GSAQVEDEEIECLVEIPTPQDYWGPVFAICIFLFSFIVPVLVISVCYSLMIRRL 237
```

>gi|16589056|gb|AAL27000.1| type II gonadotropin-releasing hormone receptor [H
Length = 379

Score = 62.0 bits (149), Expect = 1e-09
Identities = 80/332 (24%), Positives = 130/332 (39%), Gaps = 23/332 (6%)

```
Query: 38  GKEWGSFYYSFKTEQLITLWVLFVFTIVGNSVVLFSTWRRK----KKSRMTFFVTQLAIT 93
           G E  +F  + K    +T+ VLFV +  GN  VL+S  RR+    + S +      LA
Sbjct: 27  GSELPTFSAAAKVRVGVTI-VLFVSSAGGNLAVLWSVTRREPSQLRPSPVRRLFIHLAAA 85

Query: 94  DSFTGLVNILTDIIWRFTGDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYP 153
           D     V +  D  W  T  + A D                ++ ++ V + +DR  A++ P
Sbjct: 86  DLLVTFVVMPLDATWNITVQWLAVDIACRTLMFLKLMATYSAAFLPVVIGLDRQAAVLNP 145

Query: 154 MKFLQGEKQARVLIVIAWSLSFLFSIPTLIIFGKRTLS-NGEV---QCWALWPGDSYW-- 207
           +    G    R L+  AW LSFL + P L +F   T+   G V   QC       + W
Sbjct: 146 LGSRSG---VRKLLGAAWGLSFLLAFPQLFLF--HTVHUAGPVPFTQCVTKGSFKAQWQE 200

Query: 208 TPYMTIVAFLVYFIPLTIISIMYGIVIRTIWIKSKTYETVISNCSDGKLCSSYNRGLXXX 267
           T Y       ++ +PLT ++I Y  ++ ++   S+      S+   G+     +
Sbjct: 201 TTYNLFTFCCLFLLPLTAMAICYSRIVLSV---SRPQTRKGSHAPAGEFALPRSFDNCPR 257

Query: 268 XXXXXXXXXXXXXLAFICCWSPYXXXXXXXXXXXXPDTQERFYASVIIQNLPALNSAINP 327
                      L FI CW+PY              T+     S I+  L  LN+ ++P
Sbjct: 258 VRLRALRLALLILLTFILCWTPYYLLGMWYWFSPTMLTEVPPSLSHILFLLGLLNAPLDP 317

Query: 328 PIYCVFSSSISFPCREQRSQDSRMTFRERTER 359
            +Y  F    +  CR    + S  + +E + R
Sbjct: 318 LLYGAF----TLGCRRGHQELSIDSSKEGSGR 345
```

>gi|27435794|gb|AAO13224.1| KOR-3A splice variant [Homo sapiens]
Length = 341

Score = 62.0 bits (149), Expect = 2e-09
Identities = 46/169 (27%), Positives = 80/169 (47%), Gaps = 10/169 (5%)

```
Query: 75  WRRKKKSRMTFFVTQLAITDSFTGLVNIL--TDIIWRFTGDFTAPDXXXXXXXXXXXXX 132
           W  K K+    ++  LA+ D+   L      TDI+  F   +   +
Sbjct: 49  WHTKMKTATNIYIFNLALADTLVLLTLPFQGTDILLGF---WPFGNALCKTVIAIDYYNM 105

Query: 133 XASTYVLVSLSIDRYHAIVYPMKFL--QGEKQARVLIVIAWSLSFLFSIPTLIIFGKRTL 190
             ST+ L ++S+DRY AI +P++ L  +    +A+ + V  W+L+ +  +P  I+ G   +
Sbjct: 106 FTSTFTLTAMSVDRYVAICHPIRALDVRTSSKAQAVNVAIWALASVVGVPVAIM-GSAQV 164

Query: 191 SNGEVQCWALWPG-DSYWTPYMTIVAFLVYFI-PLTIISIMYGIVIRTI 237
            + E++C    P    YW P   I  FL  FI P+ +IS+ Y ++IR +
Sbjct: 165 EDEEIECLVEIPTPQDYWGPVFAICIFLFSFIVPVLVISVCYSLMIRRL 213
```

>gi|88267|pir||JQ1059 neurokinin 2 receptor - human
gi|189222|gb|AAB05897.1| neurokinin-2 receptor
Length = 398

Score = 61.6 bits (148), Expect = 2e-09
Identities = 68/316 (21%), Positives = 121/316 (38%), Gaps = 49/316 (15%)

```
Query: 42  GSFYYSFKTEQLITLWV-----LFVFTIVGNSVVLFSTW-RRKKKSRMTFFVTQLAITD- 94
           G+  +S  + QL  LW       L +  + GN++V++      R+ ++    +F+  LA+ D
Sbjct: 22  GTTAFSMPSWQL-ALWATAYLALVLVAVTGNAIVIWIILAHRRMRTVTNYFIVNLALADL 80

Query: 95  ---SFTGLVNIL--TDIIWRFTGDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHA 149
              +F    N +  +  IW F   F                      S Y + +++ DRY A
Sbjct: 81  CMAAFNAAFNFVYASHNIWYFGRAF------CYFQNLFPITAMFVSIYSMTAIAADRYMA 134

Query: 150 IVYPMKFLQGEKQARVLIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQCWALWPGDSYWTP 209
           IV+P +         + +I   W ++   + P    +   T+  G  +C   WP DS
Sbjct: 135 IVHPFQPRLSAPSTKAVIAGIWLVALALASPQ-CFYSTVTMDQGATKCVVAWPEDSGGKT 193

Query: 210 ---YMTIVAFLVYFIPLTIISIMYGIVIRTIWIKS-KTYETVISNCSDGKLCSSYNRGLX 265
              Y  +V  L+YF+PL ++ + Y ++  T+W ++   ++    +N    +    + + +
Sbjct: 194 LLLYHLVVIALIYFLPLAVMFVAYSVIGLTLWRRAVPGHQAHGANLRHLQAKKKFVKTM- 252

Query: 266 XXXXXXXXXXXXXXXLAFICCWSPYXXXXXXXXXXXXXPDTQERFYASVIIQN-------L 318
                        L F  CW PY                QE  Y    IQ        L
Sbjct: 253 -----------VLVVLTFAICWLPYHLYFILGSF------QEDIYCHKFIQQVYLALFWL 295

Query: 319 PALNSAINPPIYCVFS 334
              ++  NP IYC  +
Sbjct: 296 AMSSTMYNPIIYCCLN 311
```

>gi|21928731|dbj|BAC05952.1| seven transmembrane helix receptor [Homo sapiens]

Length = 398

Score = 61.2 bits (147), Expect = 3e-09
Identities = 62/294 (21%), Positives = 112/294 (38%), Gaps = 43/294 (14%)

```
Query: 59  LFVFTIVGNSVVLFSTW-RRKKKSRMTFFVTQLAITD----SFTGLVNIL--TDIIWRFT 111
           L +  + GN++V++      R+ ++    +F+  LA+ D    +F    N +  +  IW F
Sbjct: 43  LVLVAVTGNAIVIWIILAHRRMRTVTNYFIVNLALADLCMAAFNAAFNFVYASHNIWYFG 102

Query: 112 GDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAW 171
             F                    S Y + +++ DRY AIV+P +         + +I   W
Sbjct: 103 RAF------CYFQNLFPITAMFVSIYSMTAIAADRYMAIVHPFQPRLSAPSTKAVIAGIW 156

Query: 172 SLSFLFSIPTLIIFGKRTLSNGEVQCWALWPGDSYWTP---YMTIVAFLVYFIPLTIISI 228
            ++   + P    +   T+  G  +C   WP DS       Y  +V  L+YF+PL ++ +
Sbjct: 157 LVALALASPQ-CFYSTVTMDQGATKCVVAWPEDSGGKTLLLYHLVVIALIYFLPLAVMFV 215

Query: 229 MYGIVIRTIWIKS-KTYETVISNCSDGKLCSSYNRGLXXXXXXXXXXXXXXXLAFICCW 287
           Y ++  T+W ++   ++    +N    +    + + +                L F  CW
Sbjct: 216 AYSVIGLTLWRRAVPGHQAHGANLRHLQAMKKFVKTM------------VLVVLTFAICW 263

Query: 288 SPYXXXXXXXXXXXXPDTQERFYASVIIQN-------LPALNSAINPPIYCVFS 334
            PY                QE  Y    IQ        L   ++  NP IYC  +
Sbjct: 264 LPYHLYFILGSF------QEDIYCHKFIQQVYLALFWLAMSSTMYNPIIYCCLN 311
```

>gi|4503905|ref|NP_003848.1| galanin receptor 2 [Homo sapiens]
gi|6016094|sp|O43603|GALS_HUMAN Galanin receptor type 2 (GAL2-R) (GALR2)
gi|7441605|pir||JC5949 galanin receptor 2 - human
gi|2921760|gb|AAC39634.1| galanin receptor GalR2 [Homo sapiens]

gi|3170599|gb|AAC18118.1| galanin receptor subtype 2 [Homo sapiens]
gi|3642914|gb|AAC36587.1| galanin receptor type 2 [Homo sapiens]
gi|4165081|gb|AAD08671.1| galanin receptor type 2 [Homo sapiens]
gi|46575761|gb|AAH69130.1| Galanin receptor 2 [Homo sapiens]
gi|49901706|gb|AAH74914.1| Galanin receptor 2 [Homo sapiens]
gi|49902285|gb|AAH74915.1| Galanin receptor 2 [Homo sapiens]
Length = 387

Score = 60.8 bits (146), Expect = 3e-09
Identities = 49/204 (24%), Positives = 84/204 (41%), Gaps = 21/204 (10%)

```
Query: 134 ASTYVLVSLSIDRYHAIVYPM--KFLQGEKQARVLIVIAWSLSFLFSIPTLIIFGKRTLS 191
           AS++ L ++S+DRY AI YP+  + L+  + A   I + W LS LFS P L  + +  L+
Sbjct: 111 ASSFTLAAVSLDRYLAIRYPLHSRELRTPRNALAAIGLIWGLSLLFSGPYLSYYRQSQLA 170

Query: 192 NGEVQCWALWPGDSYWTPYMTIVAFLV-YFIPLTIISIMYGIVIRTIWIKSKTYETVISN 250
           N  V C   W         M I  F+  Y +P+ ++ + Y   +R +W   +  + V +
Sbjct: 171 NLTV-CHPAWSAPRRRA--MDICTFVFSYLLPVLVLGLTYARTLRYLW---RAVDPVAAG 224

Query: 251 CSDGKLCSSYNRGLXXXXXXXXXXXXXXXXLAFICCWSPYXXXXXXXXXXXXPDTQERFY 310
              +      R +                   F  CW P+             P T+  +
Sbjct: 225 SGARRAKRKVTRMILIVAAL------------FCLCWMPHHALILCVWFGQFPLTRATYA 272

Query: 311 ASVIIQNLPALNSAINPPIYCVFS 334
             ++   +   NS +NP +Y + S
Sbjct: 273 LRILSHLVSYANSCVNPIVYALVS 296
```

>gi|4507345|ref|NP_001048.1| tachykinin receptor 2; NK-2 receptor; Tachykinin (substance K receptor; neurokinin 2 receptor); tachykinin 2 receptor (substance K receptor, neurokinin 2 receptor); neurokinin 2 receptor [Homo sapiens]
gi|189135|gb|AAC31760.1| neurokinin A receptor [Homo sapiens]
gi|189142|gb|AAA60347.1| neurokinin A receptor
Length = 398

Score = 60.8 bits (146), Expect = 4e-09
Identities = 67/312 (21%), Positives = 119/312 (38%), Gaps = 49/312 (15%)

```
Query: 46  YSFKTEQLITLWV-----LFVFTIVGNSVVLFSTW-RRKKKSRMTFFVTQLAITD----S 95
           +S  + QL  LW       L +  + GN++V++      R+ ++   +F+  LA+ D    +
Sbjct: 26  FSMPSWQL-ALWAPAYLALVLVAVTGNAIVIWIILAHRRMRTVTNYFIVNLALADLCMAA 84

Query: 96  FTGLVNIL--TDIIWRFTGDFTAPDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYP 153
           F    N +  +  IW F   F                      S Y + +++ DRY AIV+P
Sbjct: 85  FNAAFNFVYASHNIWYFGRAF------CYFQNLFPITAMFVSIYSMTAIAADRYMAIVHP 138

Query: 154 MKFLQGEKQARVLIVIAWSLSFLFSIPTLIIFGKRTLSNGEVQCWALWPGDSYWTP---Y 210
            +         + +I   W ++   + P     +   T+  G  +C   WP DS       Y
Sbjct: 139 FQPRLSAPSTKAVIAGIWLVALALASPQ-CFYSTVTMDQGATKCVVAWPEDSGGKTLLLY 197

Query: 211 MTIVAFLVYFIPLTIISIMYGIVIRTIWIKS-KTYETVISNCSDGKLCSSYNRGLXXXXX 269
             +V  L+YF+PL ++ + Y ++  T+W ++   ++   +N    +    + + +
Sbjct: 198 HLVVIALIYFLPLAVMFVAYSVIGLTLWRRAVPGHQAHGANLRHLQAKKKFVKTM----- 252

Query: 270 XXXXXXXXXXLAFICCWSPYXXXXXXXXXXXXPDTQERFYASVIIQN-------LPALN 322
```

```
                    L F  CW PY                QE  Y    IQ        L   +
Sbjct: 253 -------VLVVLTFAICWLPYHLYFILGSF------QEDIYCHKFIQQVYLALFWLAMSS 299

Query: 323 SAINPPIYCVFS 334
           +  NP IYC  +
Sbjct: 300 TMYNPIIYCCLN 311
```

>gi|11323189|emb|CAC17003.1| dJ1022E24.1 (opiate receptor-like protein 1 (OPRI
Length = 292

```
 Score = 60.8 bits (146), Expect = 4e-09
 Identities = 35/107 (32%), Positives = 61/107 (57%), Gaps = 5/107 (4%)

Query: 135 STYVLVSLSIDRYHAIVYPMKFL--QGEKQARVLIVIAWSLSFLFSIPTLIIFGKRTLSN 192
           ST+ L ++S+DRY AI +P++ L  +    +A+ + V  W+L+ +  +P  I+ G   + +
Sbjct: 59  STFTLTAMSVDRYVAICHPIRALDVRTSSKAQAVNVAIWALASVVGVPVAIM-GSAQVED 117

Query: 193 GEVQCWALWPG-DSYWTPYMTIVAFLVYFI-PLTIISIMYGIVIRTI 237
            E++C    P    YW P   I  FL  FI P+ +IS+ Y ++IR +
Sbjct: 118 EEIECLVEIPTPQDYWGPVFAICIFLFSFIVPVLVISVCYSLMIRRL 164
```

>gi|19857032|sp|P21452|NK2R_HUMAN Substance-K receptor (SKR) (Neurokinin A rec
(NK-2R) (Tachykinin receptor 2)
gi|238766|gb|AAB20303.1| neurokinin-2 receptor, NK-2 receptor [human, Peptide, 3
gi|32482011|gb|AAP84358.1| tachykinin receptor 2 [Homo sapiens]
Length = 398

```
 Score = 60.5 bits (145), Expect = 4e-09
 Identities = 62/294 (21%), Positives = 112/294 (38%), Gaps = 43/294 (14%)

Query: 59  LFVFTIVGNSVVLFSTW-RRKKKSRMTFFVTQLAITD----SFTGLVNIL--TDIIWRFT 111
           L +  + GN++V++     R+ ++   +F+  LA+ D    +F    N +  +  IW F
Sbjct: 43  LVLVAVTGNAIVIWIILAHRRMRTVTNYFIVNLALADLCMAAFNAAFNFVYASHNIWYFG 102

Query: 112 GDFTAPDXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFLQGEKQARVLIVIAW 171
             F                   S Y + +++ DRY AIV+P +         + +I   W
Sbjct: 103 RAF------CYFQNLFPITAMFVSIYSMTAIAADRYMAIVHPFQPRLSAPSTKAVIAGIW 156

Query: 172 SLSFLFSIPTLIIFGKRTLSNGEVQCWALWPGDSYWTP---YMTIVAFLVYFIPLTIISI 228
            ++   + P    +   T+  G  +C   WP DS       Y  +V  L+YF+PL ++ +
Sbjct: 157 LVALALASPQ-CFYSTVTMDQGATKCVVAWPEDSGGKTLLLYHLVVIALIYFLPLAVMFV 215

Query: 229 MYGIVIRTIWIKS-KTYETVISNCSDGKLCSSYNRGLXXXXXXXXXXXXXXXXLAFICCW 287
           Y ++  T+W ++    ++    +N    +    + + +                L F  CW
Sbjct: 216 AYSVIGLTLWRRAVPGHQAHGANLRHLQAKKKFVKTM------------VLVVLTFAICW 263

Query: 288 SPYXXXXXXXXXXXXPDTQERFYASVIIQN-------LPALNSAINPPIYCVFS 334
           PY               QE  Y    IQ        L   ++  NP IYC  +
Sbjct: 264 LPYHLYFILGSF------QEDIYCHKFIQQVYLALFWLAMSSTMYNPIIYCCLN 311
```

>gi|27373028|gb|AAN87342.1| DRG kappa 1 splice variant KOR 1A [Homo sapiens]
Length = 366

```
 Score = 60.5 bits (145), Expect = 5e-09
 Identities = 48/184 (26%), Positives = 86/184 (46%), Gaps = 8/184 (4%)

Query: 58  VLFVFTIVGNSVVLFSTWRRKK-KSRMTFFVTQLAITDSFTGLVNILTDIIWRFTGDFTA 116
           V+FV  +VGNS+V+F    R  K K+     ++  LA+ D+            ++      +
Sbjct: 54  VVFVVGLVGNSLVMFVIIRYTKMKTATNIYIFNLALADALVTTTMPFQSTVY-LMNSWPF 112

Query: 117 PDXXXXXXXXXXXXXXXASTYVLVSLSIDRYHAIVYPMKFL--QGEKQARVLIVIAWSLS 174
            D                S + L  +S+DRY A+ +P+K L  +   +A  L  +A+++ +  W LS
Sbjct: 113 GDALCKIVISIDYYNMFTSIFTLTMMSVDRYIAVCHPVKALDFRTPLKAKIINICIWLLS 172

Query: 175 FLFSIPTLIIFGKRTLSNGEV-QCWALWPGDSY--WTPYMTIVAFLVYF-IPLTIISIMY 230
               I  +++ G +   + +V +C   +P D Y  W  +M I  F+  F IP+ II + Y
Sbjct: 173 SSVGISAIVLGGTKVREDVDVIECSLQFPDDDYSWWDLFMKICVFIFAFVIPVLIIIVCY 232

Query: 231 GIVI 234
           ++I
Sbjct: 233 TLMI 236
```

Get selected sequences | Select all | Deselect all

```
  Database: All non-redundant GenBank CDS
  translations+PDB+SwissProt+PIR+PRF excluding environmental samples
    Posted date:  Jul 19, 2004  1:54 AM
  Number of letters in database: 646,040,136
  Number of sequences in database:  1,933,177

Lambda     K      H
   0.326    0.138    0.439

Gapped
Lambda     K      H
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  AUTHORS   Laitinen,T., Polvi,A., Rydman,P., Vendelin,J., Pulkkinen,V.,
            Salmikangas,P., Makela,S., Rehn,M., Pirskanen,A., Rautanen,A.,
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            and Kere,J.
  TITLE     Characterization of a common susceptibility locus for
            asthma-related traits
  JOURNAL   Science 304 (5668), 300-304 (2004)
   PUBMED   15073379
  REMARK    GeneRIF: properties of GPRA make it a strong candidate for
            involvement in the pathogenesis of asthma and other IgE-mediated
            diseases; GPRA encodes isoforms produced in distinct patterns by
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            individuals
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  AUTHORS   Gupte,J., Cutler,G., Chen,J.L. and Tian,H.
  TITLE     Elucidation of signaling properties of vasopressin receptor-related
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  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 101 (6), 1508-1513 (2004)
   PUBMED   14757815
  REMARK    GeneRIF: has dual signaling properties in coupling to both G(q) and
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  AUTHORS   Vassilatis,D.K., Hohmann,J.G., Zeng,H., Li,F., Ranchalis,J.E.,
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            Bergmann,J.E. and Gaitanaris,G.A.
  TITLE     The G protein-coupled receptor repertoires of human and mouse
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 100 (8), 4903-4908 (2003)
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            and Kere,J.
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            asthma-related traits
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  REMARK    GeneRIF: properties of GPRA make it a strong candidate for
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REFERENCE   2  (residues 1 to 377)
  AUTHORS   Gupte,J., Cutler,G., Chen,J.L. and Tian,H.
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            G(S) pathways [vasopressin receptor-related receptor 1]
REFERENCE   3  (residues 1 to 377)
  AUTHORS   Vassilatis,D.K., Hohmann,J.G., Zeng,H., Li,F., Ranchalis,J.E.,
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            Bergmann,J.E. and Gaitanaris,G.A.
  TITLE     The G protein-coupled receptor repertoires of human and mouse
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34. We thank E. Ruppert, R. Montanucci, and D. Bushek for careful reading and suggestions that have improved the quality of this manuscript. We also thank J. Hatchell for his efforts in cell enumeration and construction of the oyster holding facility; B. Kay for technical help with EM; J. Mount for assistance in revised figure preparation; and T. McNutt Scott, M. Johnstone, S. Patel, and M. Justus for invaluable discussions. We gratefully acknowledge support from NSF under award no. 20020445.

Supporting Online Material
www.sciencemag.org/cgi/content/full/304/5668/297/DC1
Materials and Methods
Figs. S1 to S5
References

15 August 2003; accepted 2 March 2004

Characterization of a Common Susceptibility Locus for Asthma-Related Traits

Tarja Laitinen,[1,2] Anne Polvi,[2] Pia Rydman,[1] Johanna Vendelin,[1,2] Ville Pulkkinen,[1] Paula Salmikangas,[1] Siru Mäkelä,[2] Marko Rehn,[1] Asta Pirskanen,[1] Anna Rautanen,[4] Marco Zucchelli,[5] Harriet Gullstén,[2,5] Marina Leino,[6] Harri Alenius,[6] Tuula Petäys,[7] Tari Haahtela,[7] Annika Laitinen,[3] Catherine Laprise,[9] Thomas J. Hudson,[10] Lauri A. Laitinen,[8] Juha Kere*[2,5]

Susceptibility to asthma depends on variation at an unknown number of genetic loci. To identify susceptibility genes on chromosome 7p, we adopted a hierarchical genotyping design, leading to the identification of a 133-kilobase risk-conferring segment containing two genes. One of these coded for an orphan G protein–coupled receptor named GPRA (G protein–coupled receptor for asthma susceptibility), which showed distinct distribution of protein isoforms between bronchial biopsies from healthy and asthmatic individuals. In three cohorts from Finland and Canada, single nucleotide polymorphism–tagged haplotypes associated with high serum immunoglobulin E or asthma. The murine ortholog of GPRA was up-regulated in a mouse model of ovalbumin-induced inflammation. Together, these data implicate GPRA in the pathogenesis of atopy and asthma.

Asthma is a complex phenotype with a proven genetic component, and several projects to map susceptibility genes for asthma and related traits have been undertaken (*1*). The first published genome-wide scan in asthma suggested six tentative genetic loci, among them chromosome 7p, which was then strongly implicated in a study of Finnish and Canadian families and confirmed in West Australian families (*2–4*). To positionally clone asthma candidate genes on chromosome 7p, we studied the Finnish Kainuu subpopulation and considered three alternative hypotheses. First, that only one copy of the susceptibility allele may have survived in this population, with long-conserved haplotypes being observed, as would be consistent with some previous findings (*5–7*). Second, there might exist a founder effect, common to many European populations. This would be consistent with the common disease/common variant hypothesis, as observed for example in psoriasis (*8*, *9*). In this case, the carrier frequency should be higher than a few percent, with only a short conserved haplotype (<200 kb) detected (*8*). Third, numerous mutations might exist in the putative gene, in which case only weak or absent haplotype associations might be detectable.

To distinguish between these hypotheses, we adopted a genotyping scheme whereby we increased the density of markers used, with intermediate analyses to guide further genotyping (Fig. 1A). More specifically, if genetic association analysis suggested that a haplotype occurred in patients more often than in controls, additional markers were genotyped to either exclude or support the identity-by-descent of the haplotypes observed in unrelated patients. For the haplotypes to be identical by descent, all newly typed markers would have to be identically shared between them. The genotyping was done on 86 original genome scan families and an additional 103 trios (all together, 874 subjects) (*10*). Successive rounds of genotyping and analysis by the haplotype pattern mining (HPM) algorithm (*11*) suggested the strong association of a conserved haplotype pattern spanning between NM51 and SNP563704, separated by 46 kb (Fig. 1A). The HPM algorithm searches for allele patterns shared between several haplotypes among large sets of unrelated haplotypes.

To fully explore the genetic variation in associated haplotypes, we sequenced nonrepetitive DNA segments in this interval (from position 506,401 to 638,799 in the public sequence NT_000380; all positions are given with reference to this sequence) in one patient homozygous for the susceptibility haplotype and one control subject homozygous for the most common (nonrisk) haplotype. These sequences were then compared to the public sequence (NT_000380). Two observations emerged from these analyses: first, the patient did not reveal a single instance of heterozygosity, confirming the identity-by-descent of this chromosome segment. Second, comparison of the susceptibility

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sequence to the public sequence [and the control subject's sequence that differed from NT_000380 only by two single nucleotide polymorphisms (SNPs)] revealed 72 previously unknown SNPs and 8 deletion or insertion polymorphisms (DIPs) specifying the susceptibility haplotype (table S1).

To determine the limits of the critical region, we genotyped additional SNPs in 131 trios [304 high immunoglobulin E (IgE)–associated and 220 control chromosomes], yielding a total of 51 SNPs between 490,331 and 691,245 base pairs (bp) and an average marker density of 4 kb (tables S2 and S3). Analysis of the data by HPM revealed strong association of a conserved 133-kb pattern between 514,743 and 647,327 bp (Fig. 1A). Within this segment, there was strong linkage disequilibrium between the markers (Fig. 1B). A permutation test for association showed $P \leq 0.01$ for all 43 markers in this 133-kb segment (10,000 permutations). For comparison, the nominal P value by χ^2 association test was 0.00001 for the best associated haplotype pattern.

We subsequently genotyped two additional population samples with subjects that had been diagnosed either for asthma (from Northeastern Quebec, Canada) or high IgE (from North Karelia, Finland) and corresponding family-based controls. We used 22 SNPs among Quebec families (514 asthma-associated and 258 control chromosomes) and 29 SNPs among North Karelian families (75 high IgE–associated and 49 control chromosomes). A haplotype pattern with the same limits as in Kainuu was also identified in Quebec (Fig. 1C) and North Karelia (*12*). Most of the SNPs were shared, although some were distinct from those in Kainuu. In the three populations, 13 SNPs across the most conserved 77 kb formed seven alternative haplotypes with frequencies >2%. We next sequenced the 133-kb segment from six additional individuals (including an asthmatic subject from Quebec and a North Karelian with high IgE), each homozygous for a different haplotype. This confirmed that each of the haplotypes was different from each other in SNP composition. To assess the relationships of the haplotypes, we considered a total of 40 SNPs, and a phylogenetic analysis confirmed that the risk haplotypes were closely related and distinct from the nonrisk haplotypes in all three populations (Fig. 1D).

We next tested the hypothesis that the related haplotypes (identified either on the basis of high IgE in Kainuu or North Karelia or asthma in Quebec) together conferred risk in all three populations. The risk haplotypes could be tagged by SNP522363 (allele C, table S1) and indeed associated with significant risk ($P = 0.004$ for all data combined, all three populations contributing), consistent with the common disease/common variant hypothesis (*8*). The relative risk for high se-

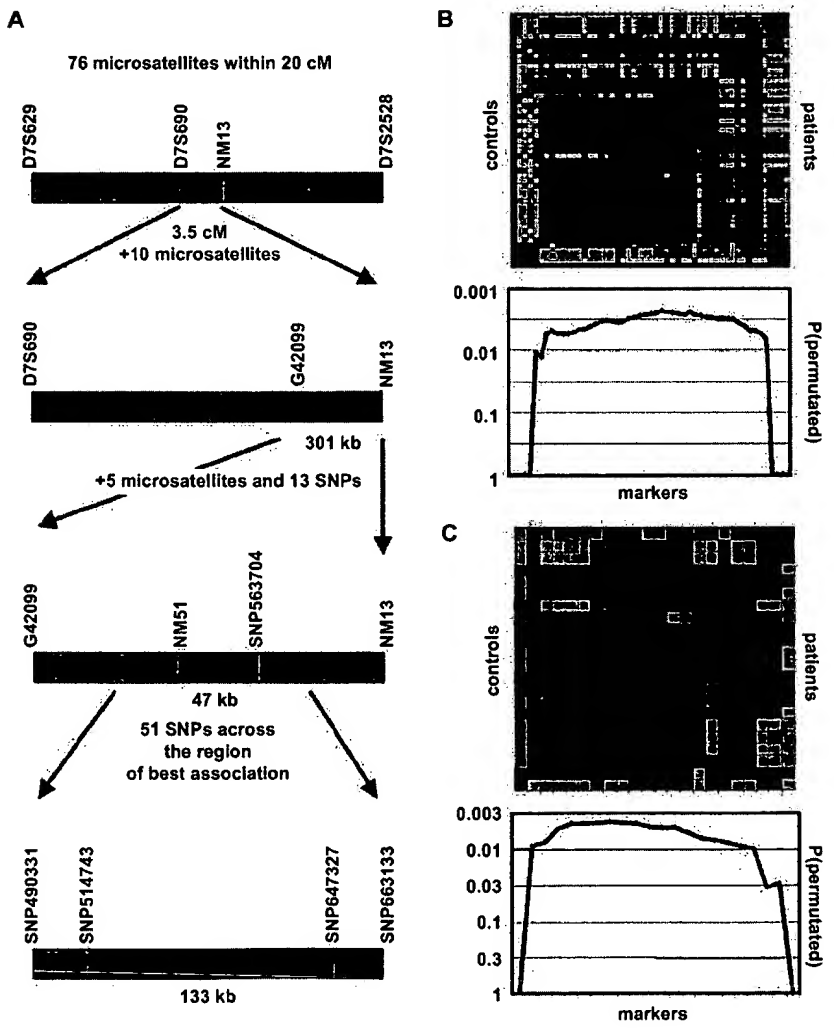

Fig. 1. (**A**) Hierarchical gene mapping strategy. The linkage region of 20 cM implicated by our genome scan (*3*) was refined by genotyping 76 microsatellite markers in families from Kainuu. We used the HPM algorithm (*11*) for finding haplotypes associated with high serum IgE. Haplotype patterns spanning 12 microsatellite markers within 3.5 cM were found associated by a permutation test implemented in HPM. At the next round of fine mapping, 10 additional microsatellites implicated a 301-kb haplotype pattern (5 markers yielded the highest associations). A further five microsatellites and 13 SNPs were genotyped next, implicating a 47-kb haplotype pattern (10 markers) between NM51 and SNP563704. All together, a 133-kb region was sequenced around this segment from a homozygous patient with asthma. Eighty polymorphisms were identified by comparison to the public genomic sequence. (**B**) Plot displaying pairwise linkage disequilibrium (LD) values between 51 markers genotyped in Kainuu trios. The markers are arranged in linear order along both axes starting from lower left corner. The scale from blue to dark red indicates LD ranging from 0 to 1, respectively. LD values shown for patients and controls are essentially identical. A segment of very strong LD between 41 markers is detected. In the lower panel, the same markers were evaluated for association to high serum IgE by a permutation test. (**C**) Plot displaying LD in French Canadian trios between 22 markers spanning the same segment as in (B). A segment of strong LD has the same boundaries as in Kainuu. In the lower panel, permutation test indicated significant association to asthma in French Canadian trios. (**D**) Phylogenetic analysis of haplotypes H1 to H7 within a 77-kb segment in Kainuu, North Karelia, and Quebec. The same seven haplotypes occur in all three populations at frequencies >2%. H4 and H5 are the most common risk-associated haplotypes in Kainuu, H7 in North Karelia, and H2 among French Canadians. H1, H3, and H6 are nonrisk haplotypes in all three populations.

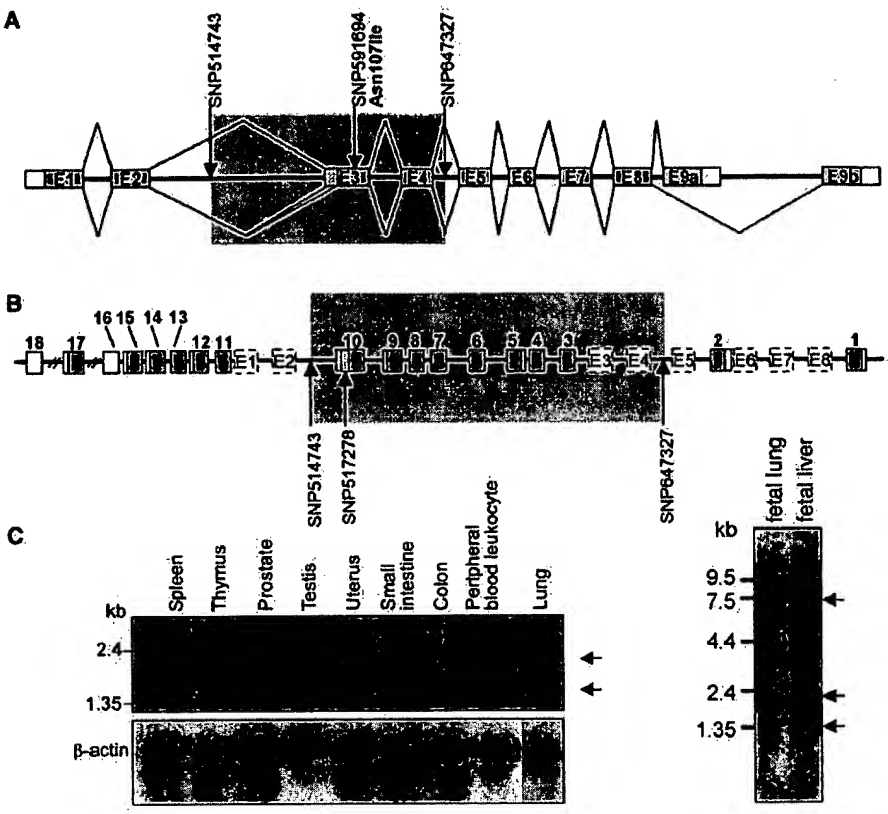

Fig. 2. Gene content around the conserved 133-kb haplotype segment (gray box). (**A**) The 133-kb segment spans from intron 2 to intron 5 of GPRA. GPRA undergoes alternative splicing with multiple variants; the three longest variants are shown (thin lines joining exons marked E1 to E9b). Exon 2 donor site may join to alternative exon 3 acceptor sites, separated by 33 bp in the same reading frame, and there are two alternative 3′ exons, 9a and 9b. Further splice variants may skip exon 3 or 4 or both, suggesting an involvement of the associated polymorphisms in regulation of splicing and protein isoform production. (**B**) In the opposite DNA strand, there is a previously unknown gene, AAA1, with at least 18 exons (numbered 1 to 18) with complex alternative splicing. AAA1 spans a total of 500 kb of genomic sequence. Eight exons of GPRA (E1 to E8) are shown for orientation. (**C**) Northern blot hybridization with a 1285-bp full-length GPRA-A cDNA probe (left) and a mixed splice variant probe for AAA1 (right). A 2.4-kb transcript is visible in all nine lanes (upper arrow) and a 1.8-kb transcript (lower arrow) in four tissues for GPRA. Several alternative transcripts are seen for AAA1 (arrows).

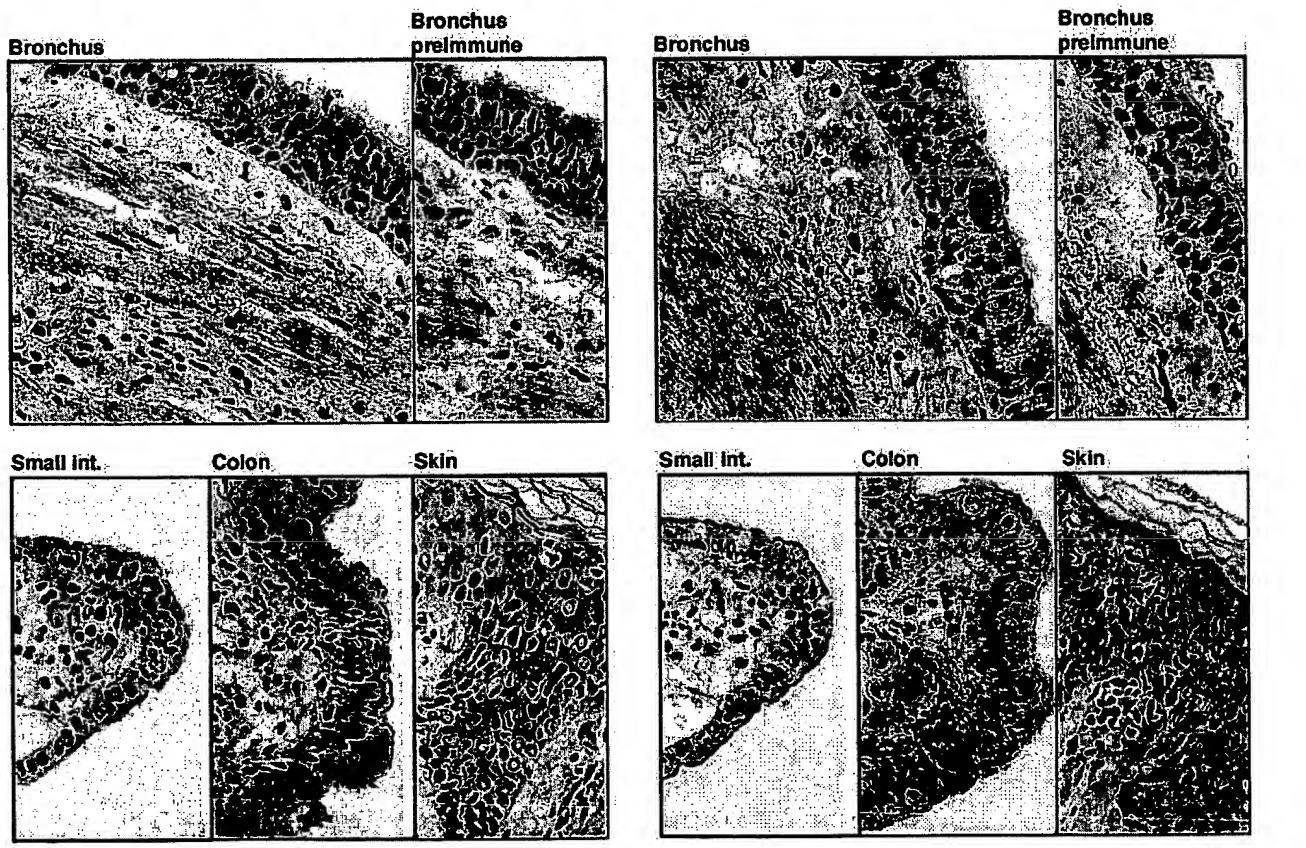

Fig. 3. Expression of the A (**left**) and B (**right**) isoforms of GPRA as assessed by immunohistochemistry. The A isoform is found in bronchial smooth muscle cells, basally in colon epithelium, and in occasional basal keratinocytes. The B isoform stains apical epithelial cells in bronchus and gut and all layers of the epidermis. Negative controls are shown with preimmune sera.

rum IgE among H4 or H5 carriers in Kainuu was 1.4 (95% confidence interval 1.1 to 1.9, $P = 0.01$), and for asthma among homozygous H2 carriers in Quebec, 2.5 (95% confidence interval 2.0 to 3.1, $P = 0.0009$). Corresponding transmission disequilibrium test yielded $P = 0.05$ for Kainuu families ($n = 86$ trios). To assess whether genetic linkage to chromosome 7p could be explained by these haplotypes, we considered parent-offspring transmissions and sibling-pair sharing of high IgE in Kainuu families (*3*). One of the risk haplotypes cosegregated in 26 of 51 transmissions (51%) and was shared in 26 of 40 sibling pairs (65%), suggesting that a majority of the linkage signal was because of the observed risk haplotypes.

These results strongly implicated the 133-kb genomic segment as a susceptibility locus for asthma-related phenotypes. To understand more precisely how the observed genetic variation might influence susceptibility, we examined the DNA segment for specific genes (*10*). Two genes were identified, one with exons 3 to 5 and the other with exons 3 to 10 lying within the susceptibility haplotype. Structures of both indicated complex alternative splicing of the mRNAs (Fig. 2 and tables S4 and S5), suggesting translation to varying protein isoforms. One of the genes (Fig. 2A) was predicted to belong to the G protein–coupled receptor family, and we named this gene GPRA (for G protein–coupled receptor for asthma susceptibility). The two main transcripts of GPRA (A and B) had alternative 3′ exons encoding proteins of 371 and 377 amino acids, respectively (GenBank AY310326 and AY310327). The sequences of all predicted isoforms of the other gene, named AAA1 (for asthma-associated alternatively spliced gene 1, GenBank AY312365 to AY312373) (Fig. 2B) showed only weak homologies to any previously identified proteins.

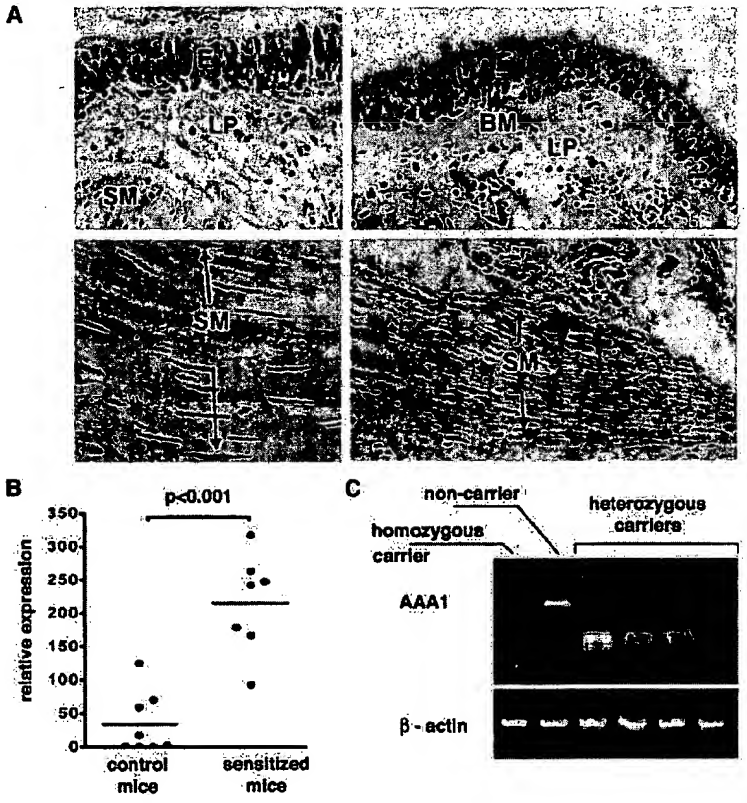

Fig. 4. (**A**) Expression of GPRA isoform B in bronchial biopsies from a healthy control (left) and an asthma patient (right). E, epithelium; BM, basement membrane; LP, lamina propria; SM, smooth muscle. (Top) The airway epithelium in the control sample shows only faint staining. In the asthma patient, the epithelium shows Goblet cell hyperplasia and basement membrane thickening typical of asthma and positive immunostaining for GPRA throughout the ciliated cells but not Goblet cells. (Bottom) The asthmatic smooth muscle stains strongly positive for GPRA isoform B, in contrast to the negative finding in control. Results are typical of 8 asthmatic and 10 control biopsies studied. (**B**) Relative expression levels of Gpra mRNA in lungs from sensitized ($n = 7$) and control ($n = 8$) mice after inhaled ovalbumin challenge. Gpra was significantly up-regulated in sensitized compared with control mice. (**C**) Variable alternative splicing for AAA1 depending on genotype. Reverse transcription polymerase chain reaction spanning exons 6 to 10b of AAA1 was performed on lymphoblast RNA samples genotyped for the susceptibility haplotype. Only noncarriers process normal amount of the exon 6-10b transcript, whereas homozygote and heterozygotes show either absent transcript or smaller splice variants. Beta-actin was used as control in parallel amplifications.

Transcripts for both genes were detected by Northern blot hybridizations (Fig. 2C). Both genes displayed coding polymorphisms in the asthma susceptibility haplotype. In GPRA, SNP591694 changed an amino acid ($Asn^{107} \rightarrow Ile^{107}$) in the first exoloop lining the putative ligand-binding pocket. Even if the position was functionally important, it was unclear how the altered protein would affect cells and tissues.

Polyclonal antibodies were raised against the different carboxyl termini of the A and B isoforms of the predicted GPRA protein (*10*). Immunohistochemical staining of bronchus, gut, and skin sections showed that the A isoform is predominantly expressed by smooth muscle cells, whereas the B isoform was predominantly detected in epithelial cells (Fig. 3). In bronchial biopsies, the isoform patterns were distinct between asthma patients and control samples (Fig. 4A). Most clearly, strong expression of the B isoform in smooth muscle cells in asthmatic airways compared with an absence of such staining in control samples. Staining for the B isoform in epithelial cells varied between healthy individuals (Figs. 3 and 4A) but was consistently stronger in the asthma samples than in controls. The A isoform showed no consistent differences. These results suggested also that one or more SNPs or DIPs in the risk haplotypes might critically alter the balance between the isoforms.

We examined next the potential role of the mouse ortholog of GPRA (Gpra) in a mouse model of ovalbumin-induced lung inflammation (*10*). In general agreement with the results in human asthma, Gpra mRNA was significantly up-regulated in mouse lung after ovalbumin tests in sensitized compared with nonsensitized mice (Fig. 4B). No ortholog for AAA1 was found in mouse and could thus not be assayed. These data support a role for GPRA in the pathogenesis of asthma and provide an animal model that may be useful to further assess the functions of this protein.

In contrast to the GPRA results, several lines of evidence suggested that AAA1 may not represent a protein-coding gene, although its expression was modified by the haplotype (Fig. 4C). Its longest open reading frame comprised only 74 potential amino acids, and in vitro translation failed to yield a stable polypeptide. Transiently transfected cells did not produce recombinant protein. Polyclonal peptide antibodies detected the antigen but no proteins in Western blots or immunohistochemistry (*12*).

Recently, a small number of candidate genes influencing susceptibility to asthma have been identified by positional cloning. However, the biochemical mechanisms linking the candidate genes to pathogenetic processes in asthma remain poorly understood (*1*, *13*–*15*). The properties of GPRA make it a strong candidate for involvement in the pathogenesis of asthma and other IgE-mediated diseases, as well as a possible drug target. GPRA might act as a receptor for an unidentified ligand. The putative ligand, iso-

forms of GPRA, and their putative downstream signaling molecules may define a new pathway critically altered in asthma. GPRA encodes isoforms that are produced in distinct patterns by bronchial epithelial cells and smooth muscle cells in asthmatic and healthy individuals. In addition, it is expressed by gut epithelia and keratinocytes of the skin, suggesting a potential role in a wider spectrum of allergic diseases.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/304/5668/300/DC1

Materials and Methods

Tables S1 to S6

4 August 2003; accepted 9 March 2004

The *Ashbya gossypii* Genome as a Tool for Mapping the Ancient *Saccharomyces cerevisiae* Genome

Fred S. Dietrich,[1,2] Sylvia Voegeli,[1] Sophie Brachat,[1] Anita Lerch,[1] Krista Gates,[3] Sabine Steiner,[1] Christine Mohr,[1] Rainer Pöhlmann,[1] Philippe Luedi,[1,2] Sangdun Choi,[4] Rod A. Wing,[4] Albert Flavier,[3] Thomas D. Gaffney,[3] Peter Philippsen[1]*

We have sequenced and annotated the genome of the filamentous ascomycete *Ashbya gossypii*. With a size of only 9.2 megabases, encoding 4718 protein-coding genes, it is the smallest genome of a free-living eukaryote yet characterized. More than 90% of *A. gossypii* genes show both homology and a particular pattern of synteny with *Saccharomyces cerevisiae*. Analysis of this pattern revealed 300 inversions and translocations that have occurred since divergence of these two species. It also provided compelling evidence that the evolution of *S. cerevisiae* included a whole genome duplication or fusion of two related species and showed, through inferred ancient gene orders, which of the duplicated genes lost one copy and which retained both copies.

The filamentous fungus *Ashbya gossypii* is currently used in industry for the production of vitamin B_2 (*1*). It is also an attractive model to study filamentous growth, because of its small genome, haploid nuclei, efficient gene targeting, propagation of plasmids, and growth on defined media (*2–8*). The *A. gossypii* genome project was initiated when conservation of gene order and orientation (synteny) to *Saccharomyces cerevisiae* was noted (*9*). We wanted to determine the complete gene repertoire for future work with this fungus, and we aimed at using the gene order information to fully explain the origin of gene cluster duplications in the *S. cerevisiae* genome that were proposed to represent relics of a whole genome doubling followed by extensive genome rearrangements (*10*, *11*).

Details on the sequencing of the *A. gossypii* genome (GenBank accession numbers AE016814 through AE016821) and annotation are available in the supporting online material. The seven chromosomes encode 4718 proteins, 199 tRNA genes, and at least 49 small nuclear RNA (snRNA) genes. The ribosomal DNA carries 40 copies of ribosomal RNA genes sequenced previously (*12*). The genome lacks transposons and subtelomeric gene repeats, and gene duplications are rare (table S6). The number of protein-coding genes is similar to the 4824 genes found in *Schizosaccharomyces pombe* (*13*), suggesting that this may be close to the minimum number of genes needed by a free-living fungus. The genome is extremely compact with an average distance between open reading frames (ORFs) of only 341 base pairs, contributing to an average protein-coding gene size of only 1.9 kb, clearly less than the 2.1-kb average gene size found in *S. cerevisiae* (*14*), the 2.5 kb found in *S. pombe* (*13*), and the 3.7 kb found in *Neurospora crassa* (*15*). The presence of only 221 introns in the entire *A. gossypii* genome, many at identical positions in *S. cerevisiae* homologs, contributes to the compact nature of this genome.

A. gossypii and *S. cerevisiae* diverged more than 100 million years ago, and their genomes differ substantially in GC content (52% for *A. gossypii* and 38% for *S. cerevisiae*). Still, for 95% of the protein-coding sequences of *A. gossypii*, we found homologs in the *S. cerevisiae* genome, the majority (4281 ORFs) at syntenic locations. Only 175 *A. gossypii* protein-coding genes showed homology but not synteny with *S. cerevisiae* genes, and 262 lack homology (table S3). Several genes with no homologs in *S. cerevisiae* have homologs in *S. pombe* (table S4), supporting the idea that they are real genes not or no longer present in *S. cerevisiae*. The annotation of the *A. gossypii* genome also identified gene functions present in *S. pombe* and *S. cerevisiae* but not in *A. gossypii* (table S5). Protein sequence conservation between syntenic homologs of *A. gossypii* and *S. cer-*

Table 1. Centromere assignments based on synteny of the genes flanking the seven *A. gossypii* and 16 *S. cerevisiae* centromeres. Roman numerals indicate the chromosome number in the respective organism. The two remaining centromeres (X and XII) have synteny with regions on chromosomes I and III, with the double break in synteny coming at the expected position of the centromere.

A. gossypii chromosomes	*S. cerevisiae* chromosomes
I	III, XIV
II	VIII, XI
III	XIII, XV
IV	V, IX
V	II, IV
VI	I, VII
VII	VI, XVI
Noncentromeric Region (I and III)	X, XII

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FTP site

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HomoloGene
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Map Viewer
OMIM
RefSeq
UniGene

Feedback
Help Desk
Corrections
About GeneRIFs

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RefSeq
Gene
Map Viewer

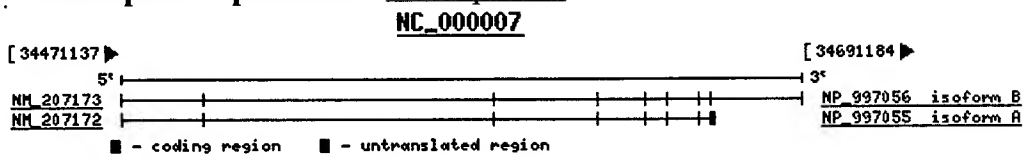
1: GPR154 G protein-coupled receptor 154 [*Homo sapiens*]

Links

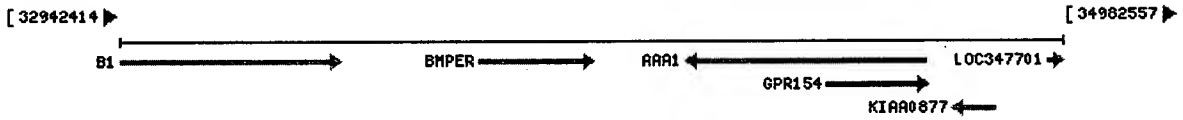
GeneID: 387129 Locus tag: HGNC:23631; MIM: 608595

updated 14-Sep-2004

Transcripts and products: RefSeq below



Genomic context: chromosome: 7; **Maps:** 7p14.3



Gene type: protein coding
Gene name: GPR154
Gene description: G protein-coupled receptor 154
RefSeq status: Provisional
Organism: *Homo sapiens*
Lineage: *Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo*
Gene aliases: GPRA; VRR1; PGR14

Bibliography: Gene References into Function (GeneRIF): **Submit** help

PubMed links

GeneRIFs:

1. has dual signaling properties in coupling to both G(q) and G(S) pathways [vasopressin receptor-related receptor 1]
2. properties of GPRA make it a strong candidate for involvement in the pathogenesis of asthma and other IgE-

mediated diseases; GPRA encodes isoforms produced in distinct patterns by bronchial epithelial & smooth muscle cells in asthma & healthy individuals

General gene information

Homology:
Mouse, Rat
Map Viewer

Phenotypes
Asthma, susceptibility to, 2 MIM: 608584

General protein information

Names: G protein-coupled receptor 154
vasopressin receptor-related receptor 1; G protein-coupled receptor for asthma susceptibility

NCBI Reference Sequences (RefSeq)

mRNA Sequence NM_207172
Source Sequence AY310326
Product NP_997055 G protein-coupled receptor 154 isoform A
Conserved Domains (1) summary
pfam00001: 7tm_1; 7 transmembrane receptor (rhodopsin family)
Location: 66 - 330 Blast Score: 254
mRNA Sequence NM_207173
Source Sequence AY310327
Product NP_997056 G protein-coupled receptor 154 isoform B
Conserved Domains (1) summary
pfam00001: 7tm_1; 7 transmembrane receptor (rhodopsin family)
Location: 66 - 330 Blast Score: 249

Related Sequences

Nucleotide		Protein
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mRNA	AY310326	AAQ76966
mRNA	AY310327	AAQ76967
mRNA	AY310328	AAQ76968
mRNA	AY310330	AAQ76970

mRNA AY310331 AAQ76971
mRNA AY310332 AAQ76972

Additional Links

UniGene Hs.453723
MIM 608595

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(*Acta Paediatr Jpn 1990*; 32: 197 ~ 200)

Urinary Arginine Vasopressin in Asthma: Consideration of Fluid Therapy

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To elucidate the role of antidiuretic hormone (ADH) on water and electrolyte balance in patients with asthmatic attacks, urinary arginine vasopressin (AVP) was assayed in 28 asthmatic patients. In a 3-year-old girl with status asthmaticus who developed a grand mal seizure in association with hyponatremia, urinary AVP levels remained high and fluctuated before convulsion; the cause of the convulsion was considered to be water intoxication due to inappropriate ADH secretion. In 19 of 28 patients with moderately severe asthmatic attacks, increases in urinary AVP levels occurred before treatment (300 ± 80 pg/ml vs. 40 ± 24 pg/ml (normal controls), $p < 0.01$); elevated AVP levels tended to fall in response to intravenous fluid therapy (appropriate ADH secretion) in 2 of 6 patients, but did not fall (inappropriate ADH secretion) in the remaining patients. It is concluded that inappropriate ADH secretion may occur in asthmatic attacks, and that in such a condition there seems to be a potential risk of water intoxication during fluid therapy, as demonstrated in the present patient.

Key Words
Urinary arginine vasopressin (Urinary AVP), Asthmatic attack, Syndrome of inappropriate secretion of ADH (SIADH), Water intoxication, Fluid therapy

Introduction

Plasma antidiuretic hormone (ADH) levels have been demonstrated to be elevated in status asthmaticus [1 – 3] and the association of syndrome of inappropriate secretion of ADH with asthmatic attack has been reported [4]. In the present study, in order to elucidate the role of ADH in water and electrolyte balance in asthmatic attacks, a detailed assay of urinary ADH (arginine vasopressin: AVP) was carried out in one patient with status asthmaticus who presented hyponatremia. Furthermore, the changes in urinary AVP in relation to treatment were investigated in other asthmatic patients.

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Patients and Methods

Assay of Urinary AVP

Urinary AVP was assayed by a sensitive radioimmunoassay according to the modified method of Rees et al [5]. The extraction procedure was performed using florisil and recovery of AVP was 96.5 ± 5.5% (mean ± SD). Dilution curves prepared from extracts of urine with and without exogenous AVP were parallel with the standard curve. The intra- and inter-assay coefficients of variation for AVP in urine were 5.0% (n = 9) and 11.0% (n = 6), respec-

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tively. The correlation coefficient of urinary AVP and osmolality (ranging from 100 to 1,400 mOsm/kg) in 28 samples was 0.93 ($p < 0.05$, Student's paired t test). The patterns of urinary AVP excretion were not different when analyzed as either pg/ml or pg/mg · creatinine; thus, we choose to present all data as pgAVP/ml. The mean urinary AVP level in 28 children aged 3 to 13 years under unrestricted fluid intake was 40 ± 24 pg/ml (mean ± SD) [6].

Patient Report

A 3-year, 4-month-old girl was admitted to our department because of a 12-hour episode of dyspnea associated with marked wheezing. She exhibited expiratory wheezing, but clinically did not appear to be dehydrated and her consciousness was clear. Her body weight was 16 kg. Chest X-ray revealed marked hyperinflation but no pneumothorax or changes suggestive of infection. Blood gases on room air demonstrated a PaO_2 of 44 mmHg, a $PaCO_2$ of 55 mmHg and a pH of 7.31. Blood chemistry disclosed a serum sodium level of 137 mmol/l, potassium 4.4 mmol/l, chloride 100 mmol/l, and urea nitrogen 2.8 mmol/l (7.8 mg/dl). Plasma osmolality was 290 mOsm/kg, urinary osmolality 572 mOsm/kg and sodium concentration, 99 mmol/l.

A commercial isotonic maintenance solution (Solita-T 3, composition: Na^+ 35 mmol/l, K^+ 20 mmol/l, Cl^- 35 mmol/l, lactate 20 mmol/l and glucose 4.3%) was intravenously administered at a rate of 80–100 ml/h (120–150 ml/kg/day) together with hydrocortisone, prednisolone and theophylline (0.8–1.0 mg/kg/h). Sixteen hours later, the patient suddenly developed tonic convulsions lasting for a few minutes, before which she had become slightly lethargic, but the patient's respiratory status had not been worsening. The serum sodium level immediately after convulsion was 122 mmol/l and serum osmolality 255 mOsm/kg. Urinary osmolality was 800 mOsm/kg and sodium concentration, 220 mmol/l. Following convulsion, the serum sodium level slowly returned to the normal range in association with intravenous fluid restriction (40 ml/h: 60 ml/kg/day) with administration of 0.9% saline solution (Na^+ 154 mmol/l, Cl^- 154 mmol/l). Assay of AVP using stored urine samples from the patient was carried out. (The details of this patient have been previously reported [7].)

Urinary AVP in Asthmatic Patients

Urinary AVP was assayed in 28 patients with asthmatic attacks aged between 3 and 16 years (mean age: 8.4 years). The clinical severity of the attacks in these patients was "moderate", i.e., obvious wheezing with respiratory difficulty, but no cyanosis or orthopnea (clinical criteria of the Japan Society for Pediatric Allergy). Clinically, these patients were not dehydrated. Urine was collected from each patient prior to the initiation of treatment (bronchodilator aerosol inhalation or subcutaneous beta-agonist injection) (first assay for urinary AVP).

In 11 of these patients who had not improved, intravenous fluid therapy (Solita-T 3, 80–120 ml/kg/24 h and theophylline) was started after voiding, and then urine was collected 3–4 hr after the initiation of intravenous fluid therapy (second assay for AVP). At this time, 4 of 6 patients who received intravenous fluid therapy still showed no clinical improvement.

Results

Details regarding water balance, serum sodium levels and urinary AVP levels during the asthmatic attack in the present patient are illustrated in Fig. 1. Changes in urinary AVP levels in the other patients with asthmatic attacks before and after treatment are shown in Fig. 2. Urinary AVP levels before treatment were elevated in 19 of the 28 patients (300 ± 80 pg/ml vs. 40 ± 24 pg/ml (normal controls), $p<0.01$, Student's t test). Urinary AVP levels tended to fall in response to fluid therapy in 2 of 6 patients, but did not fall in the remaining patients, who showed no clinical improvement in the asthmatic attacks.

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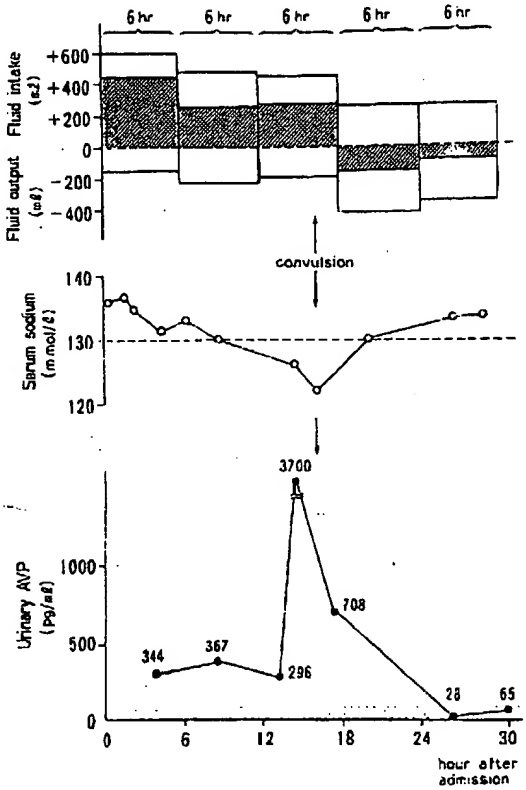

Fig. 1: Details of fluid balance, serum sodium and urinary AVP during status asthmaticus. Shaded areas indicate water balance. Dotted area indicates the normal range of urinary AVP level (upper range is mean ± 1SD for 28 controls under unrestricted fluid status).

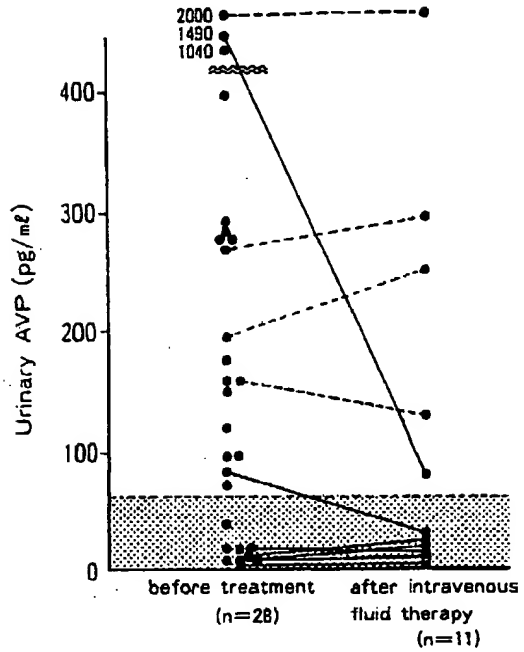

Fig. 2: Urinary AVP levels in asthmatic patients before treatment for asthmatic attacks (first assay), and the levels after intravenous fluid therapy (second assay). Solid lines indicate patients who showed clinical improvement and dotted lines, those with no clinical improvement. Dotted area indicates the normal range of urinary AVP.

Discussion

Elevated plasma ADH levels have been demonstrated in patients with status asthmaticus, and impaired water excretion during such attacks has been suggested [1, 3, 8]. It seems that there are two mechanisms of ADH secretion in asthmatic attacks. One of them may be an appropriate ADH secretion in response to dehydration (circulatory volume loss) caused by excessive sweating or inability to drink [9]. Another mechanism may be an inappropriate ADH secretion; decreased filling of the left atrium due to increased resistance to blood flow through the pulmonary bed caused by lung hyperinflation stimulates ADH secretion via the stretch receptors that exist in the left atrium [2, 9]. Hypoxemia or certain drugs (β_2-agonists, etc.) may also promote inappropriate ADH secretion [2, 8]. In the latter mechanism (inappropriate ADH secretion), ADH level may be elevated irrespective of the fluid balance or serum sodium concentration, and the levels may fall as the patient improves [3, 9].

In the present patient with status asthmaticus, the urinary AVP levels remained high and fluctuated during the hospital treatment. Therefore, the cause of convulsion, which occurred after 16 hr of positive water balance, was considered to be water intoxication (dilutional hyponatremia) due to inappropriate secretion of ADH. In this study, it also appeared that urinary AVP levels were elevated in most patients with moderately severe asthmatic attacks. Such elevation may be partly

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due to appropriate ADH secretion in response to subclinical dehydration. However, the fact that urinary AVP levels still remained high even after sufficient intravenous fluid administration seems to indicate that inappropriate ADH secretion was occurring in these patients.

Fluid therapy is important for patients with severe asthmatic attacks, but there is a potential risk of water intoxication. In this condition, not only large volumes of intravenously administered hypotonic sodium-free fluid but even isotonic fluid containing sodium at a relatively low concentration (Na^+ 35 mmol/l in this patient) may cause dilutional hyponatremia. Therefore, care should be taken not to overhydrate the patient. In patients in whom the serum sodium concentration is declining (below 130 mmol/l) during an attack, a maintenance fluid containing sodium at relatively high concentration (Na^+ 70–80 mmol/l) may be appropriate for preventing the rapid development of dilutional hyponatremia (water intoxication).

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Genetics of Vasopressin Receptors

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Membrane receptors that couple to guanine nucleotide binding protein (GPCRs) represent one of the largest families of proteins in the genome. Because of their universal distribution and multiple actions, genetic variations of GPCRs are associated with various human diseases. For instance, the clinical phenotype of congenital nephrogenic diabetes insipidus has been linked to more than 155 loss-of-function putative mutations of the arginine vasopressin (AVP) V_2 receptor, which span each and every segment of this seven-transmembrane domain receptor. These mutant receptors, which are mostly trapped in the endoplasmic reticulum, can be rescued by membrane-permeant nonpeptidic AVP receptor antagonists. An overexpression of V_1-vascular and V_3-pituitary AVP receptors has been observed in some endocrine tumors. The single nucleotide polymorphism of AVP receptors in the context of complex genetic traits is currently being investigated, and preliminary findings have been reported in arterial hypertension and autism.

Introduction

The heterotrimeric guanosine triphosphate (GTP)-binding protein (G protein)-coupled receptors (GPCRs) account for 5% of the human genome, with at least 700 GPCRs identified [1]. These plasma membrane–bound receptors recognize various extracellular signals, including peptides, amines, nucleotides, ions, and photons. Upon activation, the GPCRs couple to heterotrimeric G proteins and trigger various intracellular signaling pathways modulating numerous cellular functions, including metabolism, cell growth, and differentiation. Because of their universal distribution and multiple actions, genetic variations of GPCRs are associated with various human diseases [2,3].

The neurohypophysial antidiuretic hormone arginine vasopressin (AVP) regulates free water reabsorption, body fluid osmolality, blood volume, blood pressure, cell contraction, cell proliferation, and adrenocorticotropin hormone (ACTH) secretion via the stimulation of specific GPCRs currently classified into V_1-vascular (V_1R), V_2-renal (V_2R), and V_3-pituitary (V_3R) subtypes having distinct pharmacologic profiles and intracellular second messengers [4•].

The AVP receptors belong to the class A of GPCRs, which are structurally characterized by [5]:

- A disulfide bridge between two cysteine residues in extracellular loops (EL) 1 and 2, maintaining the proper conformation of the receptor
- A ligand-binding site that is located within the seven-transmembrane (TM) helices
- An aspartate-arginine-tyrosine (Asp[D]-Arg[R]-Tyr[Y]) sequence (DRY motif) in the proximal region of intracellular loop (IL) 2 controlling specificity of G-protein coupling
- Proline residues in the TM regions, key to receptor folding and processing
- An asparagine-proline-any amino acid-tyrosine (Asn-Pro-X-X-Tyr) motif in TM7 involved in receptor internalization and signaling
- A cysteine (two adjacent ones in the case of AVP receptors) in the C-terminal domain, which can be palmitoylated, forming a fourth IL
- A cluster of G protein–related kinase (GRK)–phosphorylated serines in the C-terminal domain, which modulates receptor internalization and rate of desensitization

In this article, the genetic variations and polymorphisms of human AVP receptors, most of which lead to loss-of-function phenotypes, are reviewed. Deciphering these mutations and linking them to clinical phenotypes has been facilitated by sequencing of AVP receptors of affected individuals, site-directed mutagenesis experiments, immunofluorescence, immunoprecipitation, and three-dimensional modeling of wild-type and mutated AVP receptors.

Arginine Vasopressin Receptor Variants and Monogenic Diseases

The most frequent mutations of human GPCRs affect the rhodopsin, thyrotropin-stimulating, and AVP V_2 receptors [1]. Most of these mutations occur within the coding region and the 5′ untranslated region. Depending on the resulting phenotype, these mutations can lead to either loss or gain-of-function.

Loss-of-Function Mutations of Arginine Vasopressin Receptors

Mutations of arginine vasopressin V_2 receptors

Congenital nephrogenic diabetes insipidus is a disease characterized by the inability to concentrate urine despite normal or elevated plasma concentrations of AVP. Affected patients present with polyuria, polydipsia, dehydration, fever, vomiting, and failure to thrive. A few cases of congenital nephrogenic diabetes insipidus have an autosomal recessive or dominant (Online Mendelian Inheritance in Man [OMIM] 222000 and 125800, respectively) mode of inheritance that is related to mutations in the aquaporin-2 gene (*AQP2*), which is located in chromosome region 12q13 and codes for the AVP-sensitive water channel. Ninety percent of patients with congenital nephrogenic diabetes insipidus are male subjects with an X-linked recessive form of the disease (OMIM 304800), who have mutations in the AVP V_2 receptor gene (*AVPR2*) located in chromosomal region Xq28, which codes for the AVP V_2 receptor [6].

The *AVPR2* is encoded by a single gene that comprises three exons and two small introns spanning approximately 2 kb. Upon AVP binding, the receptor activates G_s/adenylate cyclase, leading to the production of cyclic AMP (cAMP). In turn, cAMP activates protein kinase A, which triggers a phosphorylation cascade that promotes the translocation of the water channel, aquaporin 2, to the apical membrane of the renal tubules and collecting ducts. This sequence of events results in the reabsorption of water. Currently, more than 155 putative disease-causing *AVPR2* mutations have been identified [7–9,10•]. Fifty percent of these mutations are missense mutations. The remaining mutations include frameshift mutations caused by nucleotide deletions or insertions (27%), nonsense mutations (11%), large deletions (5%), in-frame deletions or insertions (4%), splice-site mutations (2%), and one-complex mutation. Mutations were found in all domains of the AVP V_2 receptor (N-terminus, ELs, ILs, TMs, and C-terminus), but most are located within the TMs. The classification of the mutant AVP V_2 receptors can be done after that of the low-density lipoprotein receptor, which is based on the function and subcellular localization of the mutant receptor. In this classification, type 1 mutant receptors reach the cell surface but display altered ligand-binding and signal transduction. Type 2 mutant receptors are characterized by defective intracellular transport and accumulate in a pre-Golgi compartment because of abnormal glycosyl-trimming maturation. A few mutant AVP V_2 receptors are inserted in the cell membrane, but are unable to bind AVP and/or trigger cAMP production. Most mutations of AVP V_2 receptors causing nephrogenic diabetes insipidus are type 2 mutations that lead to the intracellular retention of the mutated receptors by the endoplasmic reticulum. Immunofluorescence microscopy of mutated AVP V_2 receptor expressed in transfected cell lines have confirmed their intracellular retention. These loss-of-function mutations encompass more than mere intracellular retention of the receptors. For instance, the R137H mutated V_2R (mutation of the arginine residue within the highly conserved DRY motif) is phosphorylated and sequestered in arrestin-associated intracellular vesicles, even in the absence of agonist [11]. This prototypical example of naturally occurring GPCR signaling defect attributed to a constitutively desensitized receptor has been also observed for adrenergic and angiotensin receptors [12].

Because small molecules have been shown to influence the folding and organelle targeting of proteins, Morello *et al.* [13•] sought to determine if small nonpeptide AVP receptor antagonists could stabilize mutant AVP V_2 receptors and allow their maturation, cell-surface expression, and intracellular signaling. These authors demonstrated that small nonpeptidic cell-permeant V_2 receptor antagonists could act as pharmacologic chaperones that stabilize these mutant receptors and allow their maturation and cell-surface expression. In their seminal work, these authors showed that the cell-permeant V_2 receptor antagonist SR121463A dramatically increased cell-surface expression and rescued the function of eight mutant V_2 receptors known to produce nephrogenic diabetes insipidus. A cell-impermeant V_2 receptor peptidic antagonist could not mimic these effects and was unable to block the rescue induced by a permeant antagonist, thus suggesting that the nonpeptidic antagonist acts intracellularly, presumably by binding to and stabilizing partially folded mutant receptors. Accordingly, the small nonpeptidic V_2 receptor antagonists penetrate the cells and associate with the incompletely folded mutant receptors. This association would release the mutant receptors from the endoplasmic reticulum. The stabilized receptor would then incorporate into the cell membrane, where it could bind AVP and trigger signal transduction. This observation has practical clinical implications because nonpeptidic V_2 receptor antagonists could offer a new therapeutic strategy for the symptomatic treatment of nephrogenic diabetes insipidus linked to various mutations of the V_2 receptor. Because of the large number and diversity of mutations of V_2 receptors, this pharmacologic rescue represents a convenient alternative to gene therapy because various mutations could be rescued by the same, simple treatment. Recently, Tan *et al.* [14] studied the cellular trafficking of three mutated V_2 receptors (L292P, deltaV278, and R337X V_2Rs) that are retained intracellularly, are insensitive to AVP, and are not processed beyond initial immature glycosylation. Pharmacologic rescue of the L292P and R337X V_2R mutants by the membrane-permeant V_2R nonpeptidic antagonist SR121463B leads to mature glycosylated forms of the receptors that achieve normal basolateral surface localization.

Mutations of arginine vasopressin V_1 receptors and V_3 receptors

Loss-of-function mutations related to altered structure of the V_1-vascular and V_3-pituitary AVP receptors have not been reported to date; their identification is hampered by the absence of a defined clinical phenotype. The pheno-

typic consequences of V_1-vascular AVP receptors knockout in animal species have not been reported. Knockout of the V_3-pituitary AVP receptors in male mice resulted in markedly reduced aggression and modestly impaired social recognition [15]. These animals performed normally in other behaviors, including sexual behavior, suggesting that reduced aggression and social memory are not simply the result of a global deficit in sensorimotor function or motivation. Fos-mapping within chemosensory responsive regions suggested that the behavioral deficits in V_3R knockout mice are not due to defects in detection and transmission of chemosensory signals to the brain. These findings suggest that specific V_3R antagonists might be used in the treatment of the aggressive behavior observed in dementia and traumatic brain injuries.

Gain-of-Function Mutations

Mutations of GPCRs that lead to constitutively active forms of the receptors have been described for luteinizing hormone and thyrotropin-stimulating hormone receptors. Currently, no gain-of-function mutations of the three subtypes of AVP receptors have been reported. However, expression levels of AVP receptors were studied in various endocrine tumors secreting cortisol, because cortisol secretion in response to AVP has been reported in some patients with Cushing's syndrome due to adrenal adenoma or ACTH-independent bilateral macronodular adrenocortical hyperplasia.

Arginine vasopressin has been used as an investigative tool for the diagnosis of Cushing's syndrome because of the assumption that only patients with Cushing's disease (steroid-secreting adrenocortical tumors) display ACTH-independent cortisol response to AVP. Therefore, one can hypothesize that the responsiveness to AVP of steroid-secreting adrenocortical tumors could result from the overexpression of AVP receptors or the expression of constitutively active receptors. Consequently, Arnaldi *et al.* [16] studied the putative role of AVP receptors in the phenotypic response of 26 steroid-secreting adrenocortical tumors responsible for Cushing's syndrome (19 adenomas and 7 carcinomas) by using quantitative reverse-transcriptase polymerase-chain reaction (RT-PCR) for each of the AVP receptor subtypes. They observed that the V_1R messenger RNA (mRNA) was detected in normal adrenal cortex and in all tumors. The level of expression seemed to be higher in patients who responded to the AVP stimulation test in vivo. RT-PCR of the other AVP receptors showed a much lower signal for the V_2R and none for the V_3R. These investigators concluded that the AVP V_1R gene is expressed in normal and tumoral adrenocortical cells. High and not ectopic expression occurred in a minority of tumors that were directly responsive to AVP stimulation tests. The expression of constitutively active receptors by these tumors has not been ruled out, because direct sequencing of the receptors in these tumors was not performed. Another group of investigators also explored the expression level of V_1Rs in ACTH-independent macronodular adrenal hyperplasia [17•]. They found high levels of expression, nearly 10 times the control levels, in all four tissue samples examined. Nucleotide sequencing of the V_1R gene from one of these cases did not find any mutation in the coding region.

Similarly, using RT-PCR, De Keyser *et al.* [18] studied the expression of the AVP V_3R in tumors of pituitary and nonpituitary origin, because corticotroph cells are the main site of expression of this receptor. The V_3R mRNA was detected in 17 of 18 ACTH-secreting pituitary adenomas and in six of six normal pituitaries. A very faint V_3R signal was detected in growth hormone- and prolactin-secreting adenomas. Six of eight bronchial carcinoid tumors producing ACTH had high levels of V_3R mRNA, whereas only a very faint signal was detected in six of eight nonsecreting bronchial carcinoid tumors. These findings suggest that the V_3R gene is a marker of the corticotroph phenotype, whether it is of pituitary or nonpituitary origin. The functional or causal role for the V_3R in ACTH-secreting remains to be established. If it contributes to an increase in pro-opiomelanocortin (*POMC*) gene expression and/or ACTH secretion, it might be through overexpression of the wild-type receptor, expression of a constitutively active receptor, or chronic receptor stimulation via the autocrine or paracrine action of locally produced AVP. Other investigators also found that the V_3R appears to be overexpressed in ACTH-secreting tumors of pituitary or ectopic origin [19]. Mutations of the V_3R genes were searched for by PCR, single-strand conformation polymorphism (not by direct nucleotide sequencing), and none was found in the 12 tumors examined. To understand better the consequences of V_3R overexpression, Rene *et al.* [20•] generated transgenic mice expressing the human V_3R in the pituitary under the control of the rat POMC promoter (POMV3 mice). In vitro ACTH release in response to AVP was significantly increased. In vivo, basal circulating ACTH concentrations were normal, and corticosterone concentrations were moderately increased, but not to a level sufficient to produce hypercortisolism. These $POMV_3$ mice responded in the same manner as control mice to a 20-minute acute restraint stress with similar maximal increase of ACTH and corticosterone. These authors suggested that overexpression of V_3Rs in human pituitary corticotrope tumors contributes to increased response to AVP as well as their glucocorticoid resistance.

Arginine Vasopressin Receptor Variants and Complex Diseases

In addition to mutations responsible for monogenic forms of human diseases, GPCR variants have been shown to be associated with human complex diseases and traits. For instance, polymorphisms of the beta2-adrenergic receptor have been shown to be linked to asthma and the response to therapeutic agents used to treat asthma. Furthermore, as

GPCRs represent the targets for more than 50% of current drugs, it is reasonable to assume that the interindividual variability in therapeutic response to agonists or antagonists directed against these GPCRs is partly related to genetic polymorphism. Extensive analyses of single nucleotide polymorphisms (SNPs) are currently in progress and should delineate the role played by naturally occurring mutations of GPCRs in the pathophysiology of complex human diseases and traits. In a recent study, Small *et al.* [21] studied the nucleotide sequence variability of the promoter, 5'-, 3'-untranslated coding regions (UTR), coding regions, and intron-exon boundaries of 64 GPCR genes in an ethnically diverse group of 82 subjects. Of the 675 single-nucleotide variations found, 61% occurred in more than 1% of the population sample. Most of these are located in the 5'-UTR and coding regions. The prevalence of nonsynonymous coding SNPs was high, with 65% of GPCR genes having at least one. Intron-containing genes had half as many nonsynonymous coding SNPs compared with intronless genes, suggesting that coding regions provide sites for variation when introns are not available. Gene variability was most prominent in the TMs (38%) and the ILs (24%). Phosphoregulatory domains, particularly the carboxy terminus, often the site for agonist-promoted phosphorylation by G-protein–coupled receptor kinases, were the least polymorphic (8%). These data support the need to investigate genetic variability of GPCRs.

Polymorphism of Arginine Vasopressin V_1 Receptors

Because AVP modulates body fluid osmolality, blood volume, blood pressure, vascular tone, and cell proliferation via its potent antidiuretic, vasoconstrictor, and mitogenic actions, it might contribute to the pathogenesis of arterial hypertension, heart failure, and atherosclerosis. We recently reported the structure and functional expression of the human V_1R cyclic DNA and described the genomic characteristics, tissue expression, chromosomal localization, and regional mapping of the human V_1R gene, *AVPR1A* [22]. To test whether V_1R is a marker for human essential hypertension, we sequenced the human *AVPR1A* gene and its 5' upstream region and found several DNA microsatellite motifs [23•]. One $(GT)_{14}$-$(GA)_{13}$-$(A)_8$ microsatellite is located 2983 bp downstream of the transcription start site, within a 2.2 kbp intron interrupting the coding sequence of the receptor. Three other microsatellites are present in the 5' flanking DNA of the *AVPR1A* gene: a $(GT)_{25}$ dinucleotide repeat, a complex $(CT)_4$-TT-$(CT)_8$-$(GT)_{24}$ motif, and a $(GATA)_{14}$ tetranucleotide repeat located, respectively, 3956 bp, 3625 bp, and 553 bp upstream of the transcription start site. Analysis of these polymorphisms in 79 hypertensive and 86 normotensive subjects for the $(GT)_{14}$-$(GA)_{13}$-$(A)_8$ and the $(GT)_{25}$ motifs revealed a high percentage of heterozygosity, but no difference in allele frequencies between the two groups. A linkage study using the affected sib pair method and the $(GT)_{25}$ repeat in 446 hypertensive sib pairs from 282 French, white pedigrees showed no excess of alleles sharing at the *AVPR1A* locus. No linkage was found in the subgroups of patients with early-onset hypertension (diagnosis before age 40) or severe hypertension (diastolic blood pressure ≥ 100 mm Hg or requirement for ≥ two medications). These findings suggest that molecular variants of the V_1R gene are not involved in unselected forms of essential hypertension. Although the analysis of more than 400 hypertensive sib pairs should have prevented the generation of false-negative results, one cannot exclude rare mutations affecting either the promoter or the coding sequence of the *AVPR1A* gene that could affect blood pressure. Furthermore, testing linkage with a highly polymorphic marker can lead to false-negative results if different alleles share a same common susceptibility variant. Finally, the absence of linkage could be due to a modest increase in the relative risk of the disease, or to variants of the human V_1 vascular AVP receptor gene that have a detectable phenotypic effect but are too rare to be detectable in a large, common population of hypertensive subjects. In this regard, it could be of interest to analyze an intermediate phenotype, such as salt sensitivity, that might be more directly influenced by changes in the vasopressinergic system. Several large-scale studies of candidate genes in human arterial hypertension are currently in progress and should provide a more definitive answer concerning the role of the *AVPR1A* gene polymorphism in cardiovascular diseases.

Arginine vasopressin, as well as oxytocin, has been shown to play a role in social behavior and cognitive functions. Impairment in social reciprocity is a central component of autism. In preclinical studies, AVP has been shown to increase a range of social behaviors, including affiliation and attachment. The behavioral effects of AVP and the neuroanatomical distribution of *AVPR1A* vary greatly between species with different patterns of social organization. For instance, the pattern of *AVPR1A* distribution is markedly different in the Montane and prairie voles. Because the 5' flanking region differs greatly between species, one can hypothesize that mutations affecting human *AVPR1A* expression could affect social phenotype. Consequently, Kim *et al.* [24] explored the possibility that the human *AVPR1A* might contribute to individual variations in social behavior and be in linkage disequilibrium with autism. These authors genotyped the two upstream repeats nearest the transcription start site, the complex $(CT)_4$-TT-$(CT)_8$-$(GT)_{24}$ motif and the $(GATA)_{14}$ tetranucleotide repeat in 115 autism trios and conducted a family-based association test using the multiallelic transmission/disequilibrium test (MTDT). Single nucleotide polymorphisms were also looked for in the coding region and 2 kb of 5' flanking region of the *AVPR1A* gene. Nominally significant transmission disequilibrium was found between autism and the complex $(CT)_4$-TT-$(CT)_8$-$(GT)_{24}$ motif (MTDT χ^2 17.05, 9 df, $P = 0.048$), whereas the other microsatellite marker $(GATA)_{14}$ did not show evidence of transmission disequilibrium. The most significant transmission disequilibrium was observed for one allele of the complex

marker (MTDT χ^2 18.05, 15 df, $P = 0.26$). A total of 10 SNPs (one from the 5′ flanking region, five from the 5′ untranslated region, four from the coding region) were identified. One of the coding SNPs changed the 6th amino acid glycine (Gly) to serine (Ser). This nominally significant transmission disequilibrium between an *AVPR1A* microsatellite and autism requires confirmation, particularly because this association was not significant after Bonferroni correction. The SNPs that were identified might be useful for further association studies.

Polymorphism of Arginine Vasopressin V_2 Receptors

A common polymorphism has been identified in exon 3 of the *AVPR2* gene (c.927A/G), but the clinical significance of this polymorphism in human pathophysiology remains to be established [25].

Polymorphism of Arginine Vasopressin V_3 Receptors

To date, no polymorphism has been identified within the *AVPR3* gene.

Conclusions

The family of AVP receptors provides a good illustration of the fundamental roles of GPCRs in human physiology and pathophysiology. The example of the V_2 receptor demonstrates the complexity of genetic variations that lead to the same clinical phenotype. Considering the role played by GPCRs in signal transduction and cellular functions in every organ and system, elucidation of the genetic polymorphism of GPCRs in the near future is bound to play a significant role in drug discovery and pharmacogenetics.

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MOLECULAR PHARMACOLOGY OF HUMAN VASOPRESSIN RECEPTORS

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ABSTRACT

Vasopressin (AVP) and oxytocin (OT) are cyclic nonapeptides whose actions are mediated by activation of specific G protein-coupled receptors (GPCRs) currently classified into V_1-vascular (V_1R), V_2-renal (V_2R) and V_3-pituitary (V_3R) AVP receptors and OT receptors (OTR). The cloning of the different members of the AVP/OT family of receptors now allows the extensive molecular pharmacological characterization of a single AVP/OT receptor subtype in stably transfected mammalian cell lines.

The human V_1-vascular (CHO-V1), V_2-renal (CHO-V2), V_3-pituitary (CHO-V3) and oxytocin (CHO-OT) receptors stably expressed in CHO cells display distinct binding profiles for 18 peptide and 5 nonpeptide AVP/OT analogs. Several peptide and nonpeptide compounds have a greater affinity for the V_1R than AVP itself. V_2R peptide agonists and antagonists tend to be non-selective ligands whereas nonpeptide V_2R antagonists are potent and subtype-selective. None of the 22 AVP/OT analogs tested has a better affinity for the human V_3R than AVP itself. Several peptide antagonists do not select well between V_1R and OTR. These results underscore the need for developing specific and potent analogs interacting specifically with a given human AVP/OT receptor subtype.

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We measured thymidine uptake as an index of mitogenic activity elicited by activation of a given AVP/OT receptor subtype. Stimulation of V_1Rs, V_1Rs by AVP as well as OTRs by OT produces a dose dependent mitogenic response whereas AVP occupancy of V_2Rs leads to an anti-mitogenic response. For similar levels of expression of receptors, the mitogenic efficacy is ranked as follows: V_1Rs > V_3Rs > OTRs. Deletion of the C-terminus of the human V_1R which contains four PKC phosphorylation sites abolishes the mitogenic effect of AVP. We directly measured AVP- or OT-stimulated formation of cAMP in CHO-V1, CHO-V2, CHO-V3, and CHO-OT cells and the results suggest that only the AVP/OT receptor subtypes which do not stimulate cAMP production (V_1R, V_2Rs, and OTRs) increase thymidine uptake.

The mitogen-activated protein kinases (MAPKs) are a point of convergence for mitogenic signals triggered by several classes of cell surface receptors including the GPCRs. AVP-dependent activation of MAPKs was examined in CHO cells transfected with the various AVP receptor subtypes. Activation of all AVP receptor subtypes produces a dose-dependent phosphorylation of p42 and p44 MAPKs which peaked at 10 minutes, started to decay slowly afterwards in all cell types, but lasted for at least 2 hours. Since the various AVP receptor subtypes show a differential G protein coupling profile, stimulation of MAP kinase phosphorylation by the various types of AVP receptors suggests that different pathways are involved in the process.

In CHO-V_3 cells stably expressing low, medium or high levels of human V_3Rs (B_{max}: <10 pmol/mg, 10 to 25 pmol/mg, and 25 to 100 pmol/mg, respectively), AVP stimulation of phospholipase C, phospholipase A_2, [^{3}H]thymidine uptake, cAMP production, MAP kinases phosphorylation was a function of the receptor density. The V_3R activates several signaling pathways *via* different G proteins, depending on the level of receptor expression. The increased synthesis of DNA and cAMP levels observed in cells expressing medium and high levels of V_3Rs, respectively, may represent important events in the tumorigenesis of corticotroph cells.

INTRODUCTION

Vasopressin (AVP) and oxytocin (OT) are two neurohypophysial peptide hormones which stimulate specific G protein-coupled receptors (GPCRs) currently classified into V_1-vascular (V_1R), V_2-renal (V_2R) and V_3-pituitary (V_3R) AVP and OT receptors (OTR) (1–5). While the cardiovascular and renal effects of AVP are well described (6, 7), the central nervous system actions of AVP (where it acts as a neurotransmitter or neuromodulator of major functions including blood pressure, memory, body temperature, brain development, and release of pituitary hormones) remain to be explored further.

Over the last five years, various members of the family of human and animal AVP/OT receptors were cloned (8–18). These cloned receptors are now stably expressed in immortalized mammalian cell lines, thus allowing the comprehensive characterization of the intra-cellular signal transduction pathways coupled to the stimulation of a specific AVP/OT receptor subtype, without interference from other receptor subtypes and endogenously bound hormone.

In this chapter, we review the known cellular signal transduction pathways linked to the activation of the human V_1 vascular, V_2-renal, V_3-pituitary AVP receptors. In addition, we present new information on the ligand binding profile, mitogenic effect, and activation of kinase pathways by the human AVP/OT receptor subtypes stably expressed in mammalian cells.

MATERIALS AND METHODS

Materials

Standard reagents, unless stated otherwise, were from Sigma Chemical Co, St. Louis, MO. CHO cells were obtained from the American Type Culture Collection (Rockville, MD). Cell culture media and geneticin were from Gibco-BRL, Grand Island, NY. Fetal bovine serum was from Hyclone, Logan, UT. Iodogen (1,3,4,6-tetrachloro-3α-6α-diphenylglycoluril) was from Pierce Chemical, Rockford, IL. Restriction and modification enzymes were from Boehringer Mannheim, Indianapolis, IN or NE Biolabs, Beverly, MA. Stratagene's QuickChange site-directed mutagenesis kit was used to introduce site-specific mutations within the nucleotide sequence of AVP receptors. [^{125}I]Na (activity = 131 mCi/ml), [^{3}H]AVP (specific activity = 48 Ci/mmol), [^{3}H]OT (specific activity = 35 Ci/mmol), and [^{3}H]thymidine (specific activity = 1 mCi/ml) were obtained from NEN Dupont, Wilmington, DE. The pBluescript II phagemid KS, and XL2-Blue E. coli strain were from Stratagene, La Jolla, CA. The expression vectors pZeoSV and pcDNA3.1 and the antibiotic zeocin were from Invitrogen, San Diego, CA. Arginine vasopressin (AVP), oxytocin (OT), arginine vasotocin (AVT), lysine vasopressin (LVP), and most of the peptide V_1, V_2, V_1/V_3, and OT agonists and antagonists were from Bachem, Torrance, CA unless indicated otherwise. The nonpeptide V_1 antagonist SR 49059 (batch number MY10–075) and the nonpeptide V_2 antagonist SR121463A (batch number DPL6.152.1) were provided by Dr. C. Serradeil-Le Gal, Sanofi Recherche, Toulouse, France. The nonpeptide rat V_1 antagonist OPC 21268 (batch number 93F92M), and the nonpeptide V_2 antagonist OPC 31260 (batch number 93D96M) were provided by Dr. J.F. Liard, Otsuka America Pharmaceutical, Inc. Rockville, MD. The nonpeptide V_2 antagonist VPA-985 (batch number CL-347985–3) was provided by Dr. D. Hartupee, Wyeth-Ayerst Research, Princeton, NJ. The linear V_1 antagonists 4-hydroxy-phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 (OHPhaaGln) and tert-butylacetyl-D-Tyr(Et)-Phe-Val-Asn-Lys-Pro Arg-NH_2 (tBaa), the V_2 antagonist d(CH_2)$_5$[D-Ile^2-Ile^4-Ala-NH_2]AVP, and the linear V_1/V_3 antagonist phenylacetyl D-Tyr-Phe-Val-Asn-Arg-Pro Arg-Arg-NH_2 were provided by Dr Maurice Manning from the Medical College of Ohio in Toledo. The linear V_1 AVP antagonist phenylacetyl-D-Tyr(Et)-Phe-Val-Asn-Lys Pro-Tyr-NH_2, custom-synthesized by Bachem, Torrance, CA, was radioiodinated ([^{125}I]TyrPhaa) with the iodogen technique and purified by H.P.L.C. as previously described (19).

cDNA Constructs

The human V_1R clone was isolated by screening a human liver cDNA library as described before (9). The human V_2R clone was generated by PCR from human kidney cDNA (Clontech human kidney QUICK-Clone cDNA, #7112–1, lot #48570) using the sense primer 5′-CATCATGGGCCCACCATGCTCATGGCG-3′ (which introduces an ApaI restriction site upstream of the start codon and includes the first twelve nucleotides in the open reading frame) and the antisense primer 5′-ACACCCAGCTCAGTGAGCTG-3′ (downstream from the stop codon and a native ApaI site, including nucleotides 1347–1366 in (10). The V_3R cDNA clone we isolated previously (8) was derived from a human pituitary tumor. To rule out the possibility of mutations of this receptor sequence because of its tumoral origin, we generated by PCR the human V_3R cDNA from normal human pituitary gland cDNA (human pituitary gland QUICK-clone cDNA from Clontech, Catalog #7173–1, lot #46910). The sense primer was 5′-TGCTTGAAGTCCTCTGAACG-3′ (nucleotides -167 to -148 in (11)) and re-

verse primer was 5'-AAGACAGCACCATCCTAGGC-3' (nucleotides 1578 to 1597 in (11), downstream from the stop codon and a native SpeI site). An SpeI restriction site was introduced 140 bp upstream of the initiation codon by PCR and the SpeI-SpeI cDNA fragment was digested, purified and sequenced. The human OTR clone was generated by PCR from human ovary cDNA (Clontech human ovary QUICK-Clone cDNA, #7122 -1, lot #48870) using two sets of primers. The first set of primers was used to amplify the 5' region of the OTR: sense primer 5'-TCAACTTTAGGTTCGCCTGC-3' (nucleotides -271 to -252 in (12)) and reverse primer 5'-TCTTGAAGCTGATAAGGCCG-3' (nucleotides 659 to 678 in (12)). The second set of primers was used to amplify the 3' region of the OTR: sense primer 5'-CGGCCTTAT-CAGCTTCAAGA-3' (nucleotides 659 to 678 in (12)) and reverse primer 5'-TCATCTTCCATCATGGAGGC-3' (nucleotides 1,321 to 1,340 in (12)). The PCR reaction conditions required the addition of 4% DMSO for amplification of the GC-rich 5' region of the OTR. After ligation and digestion, the BamHI-KpnI fragment of the OTR was purified and sequenced.

cDNAs Subcloning and Sequencing

The EcoRI-EcoRI V_1R, ApaI-ApaI V_2R, SpeI-SpeI V_3R, and BamHI-KpnI OTR constructs were ligated into pBluescript II phagemid vectors prior to transformation of XL2-Blue E. coli strain. Double-stranded DNA sequencing was performed with the Taq Dye Deoxy Terminator cycle sequencing kit and a model 373A sequencer from Applied Biosystems, Foster City, CA.

Selection and Stable Expression of AVP/OT Receptors in CHO Cells

Stable transfection of CHO cells with the pZeoSV or pcDNA3.1 expression vector containing the sequence of the human V_1R, V_2R, V_3R or OTR cDNA clones we isolated as described above was performed using the calcium phosphate precipitation method (9). CHO-V_1, CHO-V_2, CHO-V_3 and CHO-OT cells were grown in medium F12 supplemented with 10% fetal calf serum, selected with the neomycin analogs geneticin or zeocin, and purified by the limiting dilution technique. Clones expressing various densities of AVP/OT receptors were studied by radioligand saturation and competition binding experiments as well as measurement of thymidine uptake and MAP kinases activation as described below.

Radioligand Binding Assays

Control and transfected CHO cells were grown to confluence in 24-well dishes and washed twice with PBS + 10 mM $MgCl_2$ + 0.2% BSA, pH 7.4. Saturation binding experiments of AVP/OT receptors of transfected CHO cells were performed in 24-well dishes in duplicate with increasing concentrations of [^{3}H]AVP ± 1 µM unlabeled AVP or of [^{3}H]OT + 1 µM unlabeled OT (9). Affinity (K_d) and capacity (B_{max}) of the AVP/OT receptors were calculated by a nonlinear least square analysis program. Protein concentration was measured with Pierce's BCA reagent using albumin as an internal standard. Competition binding experiments were performed as described before (9) using one fixed concentration of [^{125}I]TyrPhaa for CHO-V1 cells, [^{3}H]AVP for CHO-V2 cells and CHO-V3 cells or [^{3}H]OT for CHO-OT cells and increasing concentrations of unlabeled peptide and nonpeptide AVP/OT analogs (n = 3 to 5 for each analog) for 30 minutes at 30°C. IC_{50} values were derived from non linear least square analysis and K_i values were calculated by the equation of Cheng and Prusoff: $K_i = IC_{50}/(1+L/K_d)$.

[^{3}H]Thymidine Uptake

Sub-confluent monolayer cultures of CHO-V_1, CHO-V_2, CHO-V_3 and CHO-OT cells were grown in 24-well plates to measure thymidine uptake in the presence of AVP or OT as described previously (20–22). Cells were washed with 500 µl of F12 medium and grown for 72 hours in 500 µl of F12 medium supplemented with 15 mM Hepes and 0.1% BSA. Cells were treated with increasing concentrations of AVP or OT (ranging 10^{-12} to 10^{-6} M) for 18 hours, followed by incubation with 0.5 µCi of [^{3}H]thymidine for 45 minutes. The cells were subsequently transferred on ice, washed twice with 0.5 ml of ice-cold PBS, fixed with 1 ml of ice-cold 10% TCA for 30 min at 4°C, washed twice with 1 ml of ice-cold 5% TCA solution, and solubilized with 250 µl of 0.1 N NaOH-0.1% SDS. Aliquots were collected and radioactivity was measured in a scintillation counter.

Cell Proliferation Assay

Sub-confluent monolayer cultures of transfected CHO cells were grown in 96-well plates to measure cell proliferation in the presence of AVP using the CellTiter 96 AQueous one solution cell proliferation assay from Promega (Madison, WI). Cells were washed with F12 medium and grown for 72 hours in 100 µl of F12 medium supplemented with 15 mM HEPES and 0.1% BSA. Cells were treated with 10% FBS or AVP for 18 hours, followed by incubation with 20 µl of dye solution for 2 hours according to the manufacturer's instructions. Subsequently, absorbance was recorded at 490 nm wavelength using an ELISA plate reader (650 nm reference wavelength). Values recorded for baseline control (cells not stimulated by AVP or FCS) were subtracted.

Cyclic AMP Production

Sub-confluent monolayer cultures of control and transfected CHO cells were grown for 48 hours in 12-well dishes, serum-depleted in F12/HEPES media for 4 hours, followed by labeling in media containing 1 µCi/ml of [^{3}H]-adenine for 2 hours. Cells were washed and incubated in media containing 0.5 mM IBMX, in the absence or presence of 10 µM forskolin and/or different amounts of AVP for 10 min. The reaction was stopped by removal of media and addition of 0.5 ml ice cold 5% TCA containing 1.5 mM cAMP. Separation of [^{3}H]-cAMP was carried out as described by Evans et al. (23). The amount of [^{3}H]-cAMP synthesized during the incubation with agonists was expressed as a fraction of total labeled nucleotides present in the cell extracts (10^{-4} x cAMP dpm / total dpm in acid extract).

Phosphorylation of Kinases

Phosphorylation of p38, p42 and p44 MAP kinases, and SAPK/JUNK by AVP was measured in sub-confluent monolayer cultures of CHO-V_1, CHO-V_2 and CHO-V_3 cells grown in 12-well plates. Cells were serum-starved for 48 hours before the experiments. After stimulation with AVP alone or in the presence of antagonists, the cells were washed twice with ice-cold PBS and lyzed in buffer containing 50 mM Hepes-Tris, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 10 µg/ml pepstatin, 50 µg/ml bestatin, 200 µM Na_3PO_4, and 1 mM NaF. The lyzed cells were scrapped and centrifuged at 14,000 g for 20 min. The supernatants were subjected to SDS-PAGE. After electrophoresis, proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA) and immunoblotted over-

night at 4°C with the phospho specific antibodies directed against p38 MAPK (Tyr182), p44/42 MAPK (Tyr204), or SAPK/JNK (Tyr705) (New England Biolabs, Beverly, MA). Immunodetection was carried out with the ECL kit (Amersham, Arlington Heights, IL) following the manufacturer's recommendations. Quantification of kinases phosphorylation was performed by scanning densitometry of the autoradiograms with a USB SciScan 5000 automated scanning system from USD, Cleveland, OH.

Data Analysis

Nucleotide and amino acid sequences were analyzed with the computer package GeneWorks on a MacIntosh computer (IntelliGenetics, Inc. Mountain View, CA). Binding parameters (affinity, K_d and capacity, B_{max}) of AVP/OT receptors were calculated by a non-linear least square analysis program (24). Data were expressed as mean ± s.e.m.

RESULTS AND DISCUSSION

Precedents of the Signal Transduction Pathways Coupled to the Human V_1R, V_2R, V_3R, and OTR

V_1-Vascular Receptor. The V_1-vascular receptor expressed in the liver, vascular smooth muscle cells, and the testis is the product of the same gene, undergoing identical splicing (25). This V_1-vascular receptor is also expressed in several tissues or organs including blood platelets, adrenal cortex, kidney, reproductive organs, spleen, adipocytes, the brain and various cell lines (3T3, A10, WRK-1 and A7r5). AVP binding to the V_1-vascular receptor leads to the activation of phospholipases C, D, and A_2, the production of inositol 1,4,5-triphosphate and diacylglycerol, the activation of protein kinase C, the mobilization of intracellular calcium, the influx of extracellular calcium via receptor-operated Ca^{++} channels, and the activation of the Na^+-H^+ exchanger (2, 26). No stimulation of cAMP accumulation is noted after stimulation of the V_1-vascular receptor. The secondary nuclear signal mechanisms triggered by activation of V_1-vascular receptors include induction of immediate-early response genes expression and protein synthesis, leading to cellular hypertrophy and increased cell protein content (27). Indeed, activation of V_1-vascular receptors leads to a mitogenic response in vascular smooth muscle cells, 3T3 cells, renal mesangial cells, hepatocytes and adrenal glomerulosa cells. These responses are specifically blocked by V_1-vascular receptor antagonists of peptide and nonpeptide nature. The G proteins coupled to the V_1-vascular receptor are mainly members of the $G_{q/11}$ family, but also of the G_i family, as some of the signals activated by V_1-vascular receptors stimulation (e.g. phospholipase A_2 activation) are reduced by pertussis toxin pre-treatment (25). Shortly after agonist binding, AVP receptor internalization occurs and may contribute to receptor desensitization (28, 29). Recently, it was reported in rat vascular smooth muscle cells and 3Y1 fibroblasts that the stimulation of V_1-vascular receptors lead to the activation of the MAP kinase pathway (30, 31). The activation of MAP kinase is pertussis toxin-insensitive, *via* PKC-dependent and -independent pathways. The latter seems to involve the phosphatidylinositol 3-kinase (30). Phosphorylation and dephosphorylation of the V_1-vascular receptor and their role in the desensitization/resensitization processes remains to be demonstrated.

V_2-Renal Receptor. The V_2-renal receptor is expressed in the medullary portion of the kidney (as well as in the MDCK and LLC-PK_1 cell lines) where it mediates the antidi-

uretic effect of vasopressin. AVP binding to the V_2 renal receptor leads to the sequential coupling of the cholera toxin-sensitive G protein G_s, activation of adenylyl cyclase, production of cAMP and activation of protein kinase A, promoting the insertion of water channels (aquaporins) into the luminal surface of the renal collecting tubule cells (32, 33). An elegant work by Fahrenholz et al (34) suggests that enzymatic cleavage of the ligand-occupied V_2 renal receptor by a metalloprotease leads to a major alteration of the binding site contributing to the termination of signal transmission.

V_3-Pituitary Receptor. The V_3-pituitary receptor was described initially in corticotroph cells where it potentiates the release of ACTH; however, recent RT-PCR experiments suggest its presence in other tissues such as the kidney, the pancreas and the adrenal medulla (8, 15, 35, 36). Prior to the cloning and functional expression of the human pituitary V_3R, studies of the binding characteristics and signal transduction pathways activated following binding of AVP to this receptor have been hampered by its limited availability. Initial observations were made using either animal (rat, pig, sheep) freshly isolated cells (37–41) or samples of human corticotroph adenomas (42). In these studies, occupancy of V_3Rs by agonists triggered the sequential activation of phospholipase C and protein kinase C, the mobilization of intracellular free calcium, the phosphorylation of the myristoylated alanine-rich C kinase substrate and secretion of ACTH (42–44). Conflicting data regarding coupling of the V_3R to adenylyl cyclase have been reported (40, 41, 45). No information was available regarding the nature of the G-protein(s) and the kinases-phosphatases coupled to the V_3R, as well as the eventual mitogenic role of this receptor. Studies of ligand binding profile, coupling to phospholipase C and adenylyl cyclase revealed a unique pharmacological profile for this pituitary receptor, distinct from those of the V_1R and the V_2R subtypes. Thus, this AVP receptor subtype was designated as V_3 or V_{1b} (38, 46). A recent pharmacological characterization of the porcine pituitary AVP receptor with cyclic and linear peptide AVP antagonists confirmed that the pituitary and liver AVP binding sites were dissimilar, both cyclic and linear V_1 antagonists having in general a much lower affinity for the pituitary receptor than for the liver one (47). We have recently completed a comprehensive characterization of the signal transduction pathways linked to the human V_3-pituitary receptor expressed in CHO cells (48). Depending on the level of expression of the receptor, the V_3-pituitary receptor couples to members of the $G_{q/11}$ family, alone or in combination with G_i, and may also recruit G_s. Thus, the human V_3R has a pharmacological profile clearly distinct from that of the human V_1R and V_2R and activates several signaling pathways *via* different G proteins, depending on the level of receptor expression. The increased synthesis of DNA and cAMP levels observed in cells expressing medium and high levels of V_3Rs, respectively, may represent important events in the tumorigenesis of corticotroph cells (48).

OT Receptor. The OT receptor is expressed in the uterus, the mammary gland, the ovary, the brain, the kidney and lactotroph cells. OT binding to its receptor leads to phospholipase C activation, calcium mobilization and stimulation of phosphatidyl inositol turnover (49). A recent publication by Ohmichi et al. indicates that stimulation of the OT receptor of human uterine myometrial cells induces MAP kinase phosphorylation through a pertussis toxin-sensitive G protein (50). In human myometrial cells, the OT receptor activates phospholipase C_β by interacting with at least two types of G proteins, a member of the pertussis toxin-sensitive G_i family and a member of the pertussis toxin-insensitive $G_{q/11}$ family (51). The human myometrium OT receptor also couples to a 80 kDa G protein of the $G_{h\alpha}$ family which presumably activates a 69 kDa PLC (52).

Radioligand Binding Profile of the Human V_1R, V_2R, V_3R, and OTR

We carried out an extensive ligand binding characterization of the four human AVP/OT receptor subtypes stably expressed in the same mammalian CHO cell line. CHO cells do not express endogenous AVP/OT receptors and each clone tested in our experiments expressed a single AVP/OT receptor subtype without any cross-talk with the other receptor subtypes as one may observe in homologous hosts. The human V_1-vascular (CHO-V1), V_2-renal (CHO-V2), V_3-pituitary (CHO-V3) and oxytocin (CHO OT) receptors expressed in CHO cells have clearly distinct binding profiles as shown below.

Peptide Agonist and Antagonist Binding Profile (Table I) As expected, AVP is the endogenous ligand having the highest affinity for the human V_1R, V_2R, and V_3R, whereas OT is the endogenous ligand with the highest affinity for the human OTR. The other endogenous ligands for different species (LVP and AVT) are less potent for human receptors.

Competition binding experiments done with CHO-V1 cell line indicates that all the peptide V_1 antagonists tested in our experiments display a high affinity for the V_1R. Several compounds have a greater affinity than AVP itself (K_i = 1.7 nM), the V_1 antagonist 4OH-Phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 displaying the greatest affinity for the V_1R (K_i = 0.5 nM).

The two peptide V_2-renal agonists dDAVP and dVDAVP have a good affinity for the human V_2R (K_i = 2.7 and 0.8 nM respectively), but they are not very selective as they also bind to the human V_1R and V_3R with dissociation constants in the 10 to 25 nM range. Similarly, the two peptide V_2-renal antagonists tested in our experiments (d$(CH_2)_5$[D-Ile^2-Ile^4-Ala-NH_2]AVP and d$(CH_2)_5$[D-Ile^2-Ile^4-Arg^8-Ala^9-NH_2]AVP) show a better affinity for the human V_1R (K_i = 53 and 30 nM) and OTR (K_i = 67 and 33 nM) than for the human V_2R (K_i = 88 and 76 nM). None of the V_1-vascular antagonists tested has a good affinity for the V_2R. Both the V_1/V_3 and the OT peptide antagonists have a weak affinity for the V_2R.

The two "V_3 antagonists" we tested (Phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-NH_2 and Deamino^1(D-3-(Pyridyl)Ala^2-Arg^8)-Vasopressin) in fact exhibit a significantly better affinity for the V_1R than for the V_3R, thus confirming the current lack of potent and selective AVP V_3 antagonists. None of the 22 AVP/OT analogs tested in our experiments displayed a better affinity for the human V_3R than the endogenous ligand AVP itself. OT, the other endogenous ligand in humans, has a very low affinity for the V_3R (K_i = 1,782 nM). The V_1R antagonist 4-OH-Phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg NH_2, the most potent V_1R antagonist tested in CHO-V1 cells, also displays a high affinity for the human V_3R (K_i = 2.2 nM). Since this compound can be radioiodinated, it may replace tritiated AVP as a radioligand with high specific activity to carry out further ligand binding characterization of the human V_3R. Recently, Barberis et al. reported that the same compound displays also a high affinity for the rat V_3R (K_i = 4.5 nM) and blocks AVP-induced ACTH release in cultured rat pituitary cells (53). The linear V_1/V_3 antagonist Phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg Arg-NH_2, which displays the highest affinity for the porcine V_3R (K_i = 37.2 nM) among AVP analogs recently available (47), also exhibits a reasonable affinity for the human V_1R in our studies (K_i = 16 nM); however it behaves as a more potent V_1R analog in both species. Similarly, Deamino^1(D-3-(Pyridyl)Ala^2-Arg^8)-Vasopressin, which was reported to block the rat pituitary V_3R, displays a ten-fold higher affinity for the human V_1R (K_i = 4.3 nM) than for the human V_3R (K_i = 50 nM). The availability of the CHO-V3 cells should facilitate the screening and development of potent and selective ligands for the human V_3R.

Table 1. Affinity (K_i in nM) of AVP/OT peptide analogs for the human V_1-vascular, V_2-renal, V_3-pituitary AVP, and OT receptors stably expressed in CHO cells

Compound	V_1-vascular	V_2-renal	V_3-pituitary	OT
Endogenous hormones				
AVP	1.7	1.1	1.1	16
AVT	5.0	6.2	10	1.9
Oxytocin	64	167	1,782	0.9
LVP	2.3	3.3	2.9	25
V1 antagonists				
d(CH_2)$_5$Tyr(Me)AVP	1.6	82	359	3.0
Phenylacetyl-D-Tyr(Et)-Phe-Val-Asn-Lys-Pro-Tyr-NH_2	2.4	1,805	798	13
Phenylacetyl D-Tyr(Et)-Phe-Gln-Asn-Lys-Pro-Arg-NH_2	0.8	302	31	5.3
4-OH-Phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2	0.5	428	2.2	0.4
Phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2	0.8	282	9.4	1.14
(Dan-D-Tyr(Et)Phe-Val-ASn-Lys-Pro-Arg-NH_2	0.8	54	364	1.76
V2 agonists				
dDAVP	21	2.7	22	89
dVDAVP	10	0.8	25	290
V2 antagonists				
d(CH_2)$_5$[D-Ile^2-Ile^4-Ala-NH_2]AVP	53	88	541	67
d(CH_2)$_5$[D-Ile^2-Ile^4-Arg^6-Ala^9-NH_2]AVP	30	76	650	33
V1/V3 antagonists				
Phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-NH_2	0.6	259	16	49
Deamino1(D-3-(Pyridyl)Ala^2-Arg^8)-Vasopressin	4.3	6,422	50	192
Oxytocin antagonists				
d(CH_2)$_5$[O-Me-Tyr^2-Thr^4-Orn^8]Vasotocin	7.6	5,964	28,700	0.6
d(CH_2)$_5$[O-Me-Tyr^2-Thr^4-Orn^8-Tyr^9-NH_2]Vasotocin	3.9	929	10,229	0.5

Affinity constants (Ki in nM) were obtained using the V_1-vascular ligand [^{125}I]Phenylacetyl-D-Tyr(Et)-Phe-Val-Asn-Lys-Pro-Tyr-NH_2 for the V_1R, [^{3}H]AVP for the V_2R and V_3R, and [^{3}H]OT for the OTR. Each value represents the mean of 3 to 8 independent experiments.

Examination of the ligand binding profile of the CHO-OT cell line confirms that the two OT antagonists tested have an excellent affinity for the human OT receptor (K_i = 0.6 and 0.5 nM), but their affinity for the human V_1R is also quite good (K_i = 7.6 and 3.9 nM). Among the V_1R antagonists, the affinity of the reference peptide compound d(CH$_2$)$_5$Tyr(Me)AVP for the human OTR is good (K_i = 3 nM). By the same token, several linear V_1R antagonists display a good affinity for the human OTR, including the V_1 antagonist4OH-Phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH$_2$ (K_i = 0.4 nM). Finally, the V_2 analogs and V_3 antagonists have a poor affinity for the human OT receptor.

Nonpeptide Agonist and Antagonist Binding Profile (Table II). The nonpeptide compound OPC21268, which was initially characterized as a V_1 antagonist in the rat (K_i = 140 nM) (54), displays a weak affinity for the human V_1R as well as for the V_2R and V_3R, but displaces OT from the OTR (K_i = 185 nM) and therefore can be used as a lead compound to develop potent and selective OT nonpeptide antagonists. The nonpeptide V_1 antagonist SR49059 has an excellent affinity for the human V_1R (K_i = 1.1 nM) with a greater than 100 fold selectivity for the V_1R versus the V_2R and V_3R. However, its affinity for the human OTR expressed in CHO cells is quite good (K_i = 15 nM). The nonpeptide compound OPC31260, which was found to be a V_2 antagonist in the rat (IC_{50} = 14 nM) (55), also displays an acceptable affinity and selectivity for the human V_2-renal receptor (K_i = 12 nM). The two other nonpeptide V_2 antagonists we tested (VPA-985 and SR121463A) have an excellent affinity and specificity for the human V_2R. This selectivity of the nonpeptide compounds for the human V_2R represents an advantage over the peptide compounds and the three nonpeptide V_2 antagonists mentioned above are currently being tested in clinical trials. The five nonpeptide V_1R or V_2R antagonists tested in our cell lines have a low affinity for the human V_3R, thus underscoring the need for developing a specific and potent nonpeptide V_3R antagonist. Except for SR49059, their affinity for the human OTR is also poor.

The lack of selectivity of some compounds for the human AVP/OT receptor subtypes, together with species-related differences (56), may explain the discrepancies regarding the role of these subtypes in various signaling pathways. For instance, the vasodilatory action of pharmacologic doses of dDAVP or dVDAVP have been thought to arise from the activation of putative endothelial receptors of the V_2 subtype; however conflicting results with various antagonists tested may suggest the participation of other receptor subtypes (57, 58). These results call for the development of specific and potent analogs interacting with the various human AVP/OT receptor subtypes. The availability of mammalian cell lines stably transfected with each subtype of human AVP/OT receptors will undoubtedly facilitate the screening of such compounds.

Table II. Affinity (K_i in nM) of AVP nonpeptide antagonists for the human V_1-vascular, V_2-renal, V_3-pituitary AVP, and OT receptors stably expressed in CHO cells

Compound	V_1-vascular	V_2-renal	V_3-pituitary	OT
V_1 antagonists				
OPC21268	8,800	32,975	No displac.	185
SR 49059	1.1	119	129	15
V_2 antagonists				
OPC31260	142	12	14,254	1,561
VPA-985	147	0.86	No displac.	193
SR121463A	7,375	1.53	>15,500	2,395

Affinity constants (K_i in nM) were obtained using the V_1-vascular ligand [^{125}I]Phenylacetyl-D-Tyr(Et)-Phe-Val-Asn-Lys-Pro-Tyr-NH$_2$ for the V_1R, [^{3}H]AVP for the V_2R and V_3R, and [^{125}I]OT for the OTR. Each value represents the mean of 3 to 8 independent experiments.

Stimulation of DNA Synthesis by AVP/OT in CHO Cells Expressing the Human V_1R, V_2R, V_3R, or OTR

The V_1-vascular receptor has been shown to elicit mitogenic responses, including stimulation of DNA synthesis, expression of oncogenes, and cell proliferation (4). The influence of other AVP/OT receptor subtypes on these parameters is not well known. To begin to unravel this issue, we measured thymidine uptake in CHO cells stably transfected with the AVP V_1Rs (CHO-V1 cells, B_{max} = 12.4 pmol/mg of protein), V_2Rs (CHO-V2 cells, B_{max} = 25 pmol/mg of protein), V_3Rs (CHO-V3 cells, B_{max} = 17.9 pmol/mg of protein) and OTRs (CHO-OT cells, B_{max} = 22.6 pmol/mg of protein), as an index of mitogenic activity elicited by activation of a given AVP/OT receptor subtype. Cells were stimulated with either increasing AVP or OT concentrations (Fig. 1) or 10% fetal bovine serum. AVP produced a dose-dependent increase of thymidine uptake in CHO-V1 (E_{max} = +334%, EC_{50} = 1.30 nM) and CHO-V3 cells (E_{max} = +284%, EC_{50} = 2.01 nM). In CHO-V2 cells transfected with the human V_2 receptor, which couples to adenylyl cyclase, AVP triggered a reduction of thymidine uptake reaching its nadir at 0.1 nM AVP. OT produced a dose-dependent increase of thymidine uptake in CHO-OT cells (E_{max} = +205%, EC_{50} = 1.31 nM). Treatment with 10% fetal calf serum produced a +390% to +490% increase over baseline in these transfected cell lines. These data indicate that stimulation of V_1Rs, V_3Rs by AVP as well as OTRs by OT produces a mitogenic response whereas AVP occupancy of V_2Rs leads to an anti-mitogenic response. For similar levels of expression of receptors, the mitogenic efficacy can be ranked as follows: V_1Rs > V_3Rs > OTRs.

The mitogenic action of the V_1R is a well established phenomenon which has been observed for various cells expressing this AVP receptor subtype. The mitogenic property of AVP via activation of the V_1R is concentration-dependent and is the most potent among the responses noted for the different AVP receptor subtypes. The family of AVP/OT receptors is characterized by the fact that its members differ in their G protein coupling profiles. Liu and Wess have shown that mutant receptors containing the V_1R second intracellular loop sequence are capable of activating the phosphatidylnositol pathway whereas the mutant receptors containing the V_2R third intracellular loop sequence stimulate cAMP production (59). We constructed different mutants of the human V_1R as well as V_1/V_2R chimeras which were stably expressed in CHO cells. It appears that the molecular determinants of the mitogenic response of the V_1R are located in the C-terminus of the molecule which contains four PKC consensus motifs ([ST]X[RK]) at positions 382, 404, 407, and 410. The chimera made of the human V_1R containing the C-terminus fraction of the human V_2R can no longer stimulate thymidine uptake (Fig. 2). Similarly, C-terminus truncated forms of the human V_1R do not stimulate thymidine uptake as well as the wild type V_1R. It appears that the PKC motif at position 382 plays a key role in the transmission of the mitogenic signal since the V1-

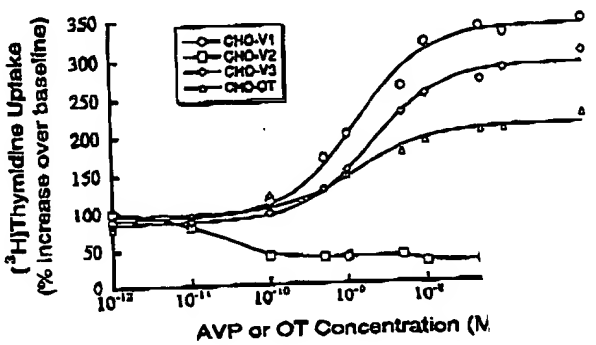


Figure 1. Stimulation of DNA synthesis by AVP or OT in CHO cells transfected with human V1, V2, V3, or OT receptors. Transfected CHO cells were grown to sub-confluence in 24-well dishes and incubated in serum-free F12 medium, pH 7.4 for 72 hours. The cells were subsequently stimulated by increasing concentrations of AVP or OT overnight, followed by incubation with [^{3}H]thymidine for 45 minutes, DNA precipitation and liquid scintillation counting.

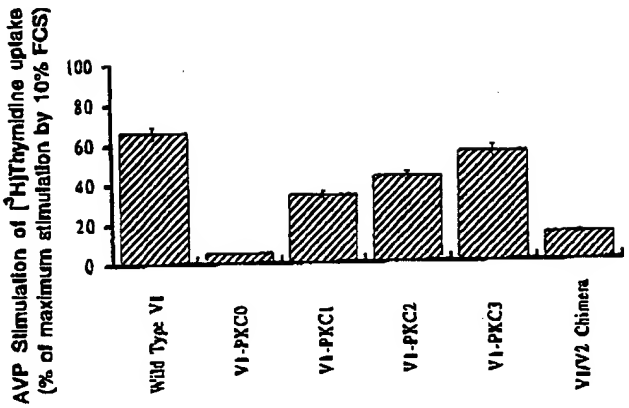


Figure 2. Stimulation of DNA synthesis by AVP in CHO cells transfected with mutated forms of the human V1 receptor. Transfected CHO cells were grown to sub-confluence in 24-well dishes and incubated in serum-free F12 medium, pH 7.4 for 72 hours. The cells were subsequently stimulated by 1 μM AVP overnight, followed by incubation with [^{3}H]thymidine for 45 minutes, DNA precipitation and liquid scintillation counting. AVP-stimulated [^{3}H]thymidine uptake results are expressed as a percent of maximum stimulation by 10% FCS.

PKC0 mutant which has no PKC site cannot produce a mitogenic signal whereas the V1-PKC1, V1-PKC2, and V1-PKC3 mutants which respectively contain 1, 2, or 3 PKC motifs do recover proportionally a mitogenic signal (Fig. 2). Assessment of AVP-induced stimulation of thymidine uptake in cell lines transfected with an AVP/OT receptor subtype will represent a convenient way to explore the mitogenic property of this specific subtype and test the influence of various pharmacological interventions. Alternatively, the influence of AVP/OT receptors stimulation on cell proliferation can be directly assessed with a colorimetric method which determines the number of viable cells (CellTiter 96 AQueous One Solution Cell Proliferation Assay from Promega) in culture in control conditions and after agonist stimulation. For instance stimulation of the V_1R by AVP produces a dose-dependent increase of cell number (EC_{50} = 1.10 nM), (Fig. 3).

The mitogenic action of the V_3R is a new finding which raises the issue of the role of V_3Rs in the development of ACTH-secreting tumors, as these tumors have been shown to express high levels of V_3Rs (36, 60).

The stimulation of thymidine uptake by OT in the CHO-OT cell line suggests that stimulation of OTRs also activates mitogenic signals. This finding deserves further attention because expression of the OTRs has been detected in human breast cancer tissue by immunohistochemistry and confirmed by Northern blotting and RT-PCR (61). By the same token, OT stimulates the proliferation of myoepithelial cells (62). This mitogenic effect of OTRs stimulation should be examined, especially in the context of breast cancer.

The reduction of thymidine uptake by AVP in the CHO-V2 cell line indicates that occupancy of V_2Rs by AVP results in decreased cell proliferation whereas stimulation of V_1Rs, V_3Rs by AVP and OTRs by OT produces a mitogenic response. This seems to suggest a negative correlation between AVP-stimulated increase in cAMP levels and cell pro-

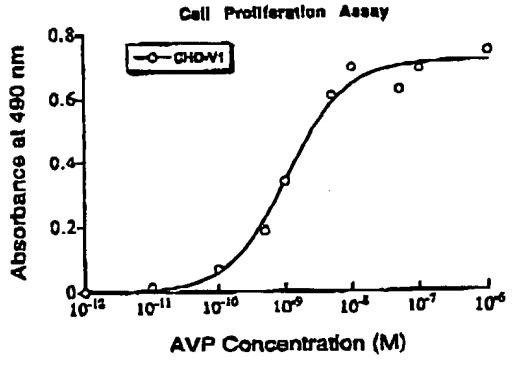


Figure 3. Stimulation of cell proliferation by AVP in CHO cells transfected with human V1 receptors. CHO-V1 cells were grown to sub-confluence in 96-well plates and incubated in serum-free F12 medium, pH 7.4 for 72 hours. The cells were subsequently stimulated by increasing concentrations of AVP overnight, followed by incubation with the novel tetrazolium compound MTS and an electron coupling reagent for 2 hours. Absorbance at 490 nm was recorded with a 96-well plate reader.

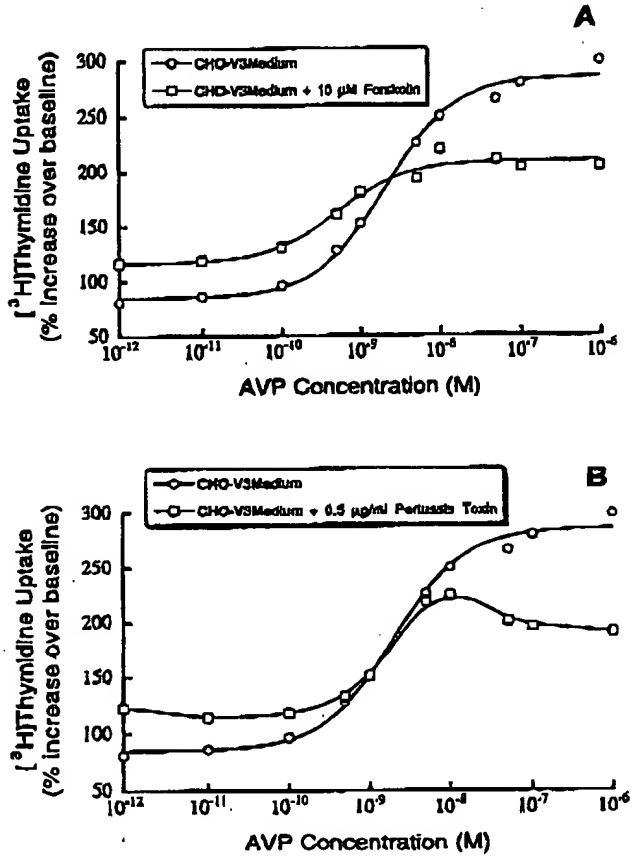


Figure 4. Effects of forskolin and pertussis toxin on AVP-dependent stimulation of DNA synthesis in CHO-V3Medium cells. CHO-V3 cells were grown to sub-confluence in 24-well dishes and incubated in serum-free F12 medium, pH 7.4 for 72 hours. Treatment with either A) forskolin (10 μM) or B) pertussis toxin (0.5 μg/ml) was performed during the last 24 h of serum depletion. The cells were stimulated overnight by increasing concentrations of AVP, followed by addition of [^{3}H]thymidine for 45 minutes, DNA precipitation and radioactivity counting.

liferation. To examine the possible role of cAMP in the mechanisms underlying the effect of AVP on thymidine uptake, we subjected CHO-V3 cells to treatment with the adenylyl cyclase activator forskolin (Fig. 4A) or the receptor-G_i protein uncoupler pertussis toxin (Fig. 4B). Pretreatment with pertussis toxin did not significantly alter the effect of fetal calf serum on thymidine uptake, whereas forskolin doubled this response (data not shown). In forskolin or pertussis toxin-treated cells, the effects of relatively high concentrations of AVP on thymidine uptake were reduced, thus suggesting that activation of adenylyl cyclase may indeed mediate the inhibitory action of high concentrations of AVP on thymidine uptake in CHO-V2 cells.

Modulation of cAMP Production by AVP/OT in CHO Cells Expressing the Human V_1R, V_2R, V_3R, or OTR

To examine the possible role of cAMP in the mechanisms underlying the effect of AVP or OT on thymidine uptake in CHO cells transfected with the various AVP/OT receptor subtypes, we directly measured AVP- or OT-stimulated formation of cAMP in CHO cells expressing the V_1Rs, V_2Rs, V_3Rs or OTRs (Table III). AVP stimulation of CHO-V1 cells did not modify cAMP formation whereas forskolin did (5 fold-increase). AVP stimulation of CHO-V2 cells produced a 9 fold increase of cAMP formation, while forskolin produced a 3-fold increase of cAMP production. In CHO-V3 cells expressing levels of receptor density similar to those of the CHO-V1 and CHO-V2 cells, AVP did not alter cAMP production. Finally, OT did not alter cAMP production in CHO-OT cells. These results suggest that stimulation of thymidine uptake by AVP/OT receptors occurs only for the subtypes which do not stimulate cAMP production.

Table III. AVP-dependent stimulation of cAMP production in CHO-V1. CHO-V2, CHO-V3 cells, and CHO-OT cells[a]

	cAMP (fold-increase over baseline)			
Agonist	CHO-V1	CHO-V2	CHO-V3	CHO-OT
Forskolin (10 μM)	5	3	5	5
AVP or OT (100 nM)	0	9	0	0

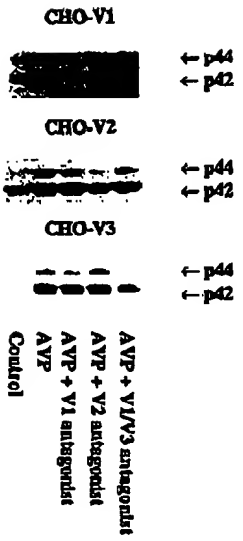
[a]Cells were pre-labeled with 0.5 μCi [^{3}H]-adenine for 2 h, followed by incubation with 0.5 mM IBMX in presence or absence of the indicated agonists, as described in Materials and Methods. The amount of cAMP formed was expressed as fold stimulation over basal.

AVP Stimulation of the MAP Kinase Pathway. The various AVP receptor subtypes show a differential G protein coupling profile. The mitogen-activated protein kinases (MAPKs) are a point of convergence for mitogenic signals triggered by several classes of cell surface receptors including the GPCRs. G_i- and G_q- coupled receptors stimulate MAPK activation *via* distinct signaling pathways (63). In transfected COS-7 cells, MAP kinases can be stimulated by G_s, G_i and G_q through participation of both α and βγ subunits (64).

AVP-dependent activation of MAP kinases was examined in CHO cells transfected with the various AVP receptor subtypes. As activation of the human OTR by OT has been recently been shown to stimulate MAP kinase activity (50), experiments with CHO-OT cell line were not performed. Figure 5 shows the immunoblotting with a phospho-specific p44/42 MAPK (Tyr204) antibody of CHO-V1, CHO-V2 and CHO-V3 cells stimulated by AVP. AVP stimulation (0.1 μM) for 10 minutes produced a significant increase in phosphorylation of p42 and p44 MAP kinases. In the CHO-V1 cells, this effect was potently blocked by an equimolar concentration of the V_1 antagonist d(CH_2)$_5$Tyr(Me)AVP. The V_2 antagonist d(CH_2)$_5$[D-Ile^2-Ile^4-Ala-NH_2]AVP and the V_1/V_3 antagonist Phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-NH_2, which are in fact nonselective analogs binding to the V_1 receptor, did reduce the effect of AVP on MAP kinases phosphorylation in CHO-V1 cells. In CHO-V2 cells, AVP also stimulated MAP kinases phosphorylation, an effect that was specifically blocked by the V_2 antagonist d(CH_2)$_5$[D-Ile^2-Ile^4-Ala-NH_2]AVP. The V_1 antagonist d(CH_2)$_5$Tyr(Me)AVP and the V_1/V_3 antagonist Phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-NH_2 did not alter AVP effect on MAP kinase in CHO-V2 cells. Finally, AVP stimulation of MAP kinase phosphorylation occurred in CHO-V3 cells and was specifically blocked by the V_1/V_3 antagonist Phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-NH_2, whereas the V_1 antagonist d(CH_2)$_5$Tyr(Me)AVP or the V_2 antagonist d(CH_2)$_5$[D-Ile^2-Ile^4-Ala-NH_2]AVP were ineffective. The phosphorylation of p42 and p44 induced by AVP in CHO-V3 cells was concentration-dependent (EC_{50} = 0.48–1.34 nM)

The extent and duration of AVP-induced phosphorylation of p42 and p44 MAP kinases were explored in CHO cells stably expressing V_1Rs, V_2Rs, or V_3Rs (Fig. 6). The phosphorylation of p42 and p44 induced by AVP stimulation (100 nM) peaked at 10 minutes and started to decay slowly afterwards in all cell types. In these transfected CHO cells, the effect of AVP lasted for at least 2 hours.

Stimulation of MAP kinase phosphorylation by the various types of AVP receptors suggests that different pathways are involved in the process. The V_1Rs presumably act through activation of $G_{q/11}$, PLC, PKC and PI_3Kinase (30, 48). Activation of MAP kinase phosphorylation by the V_2R, presumably through activation of G_s, is interesting in view of the reported inhibition of MAP kinases by cAMP (64). The role of G_α in regulating cell



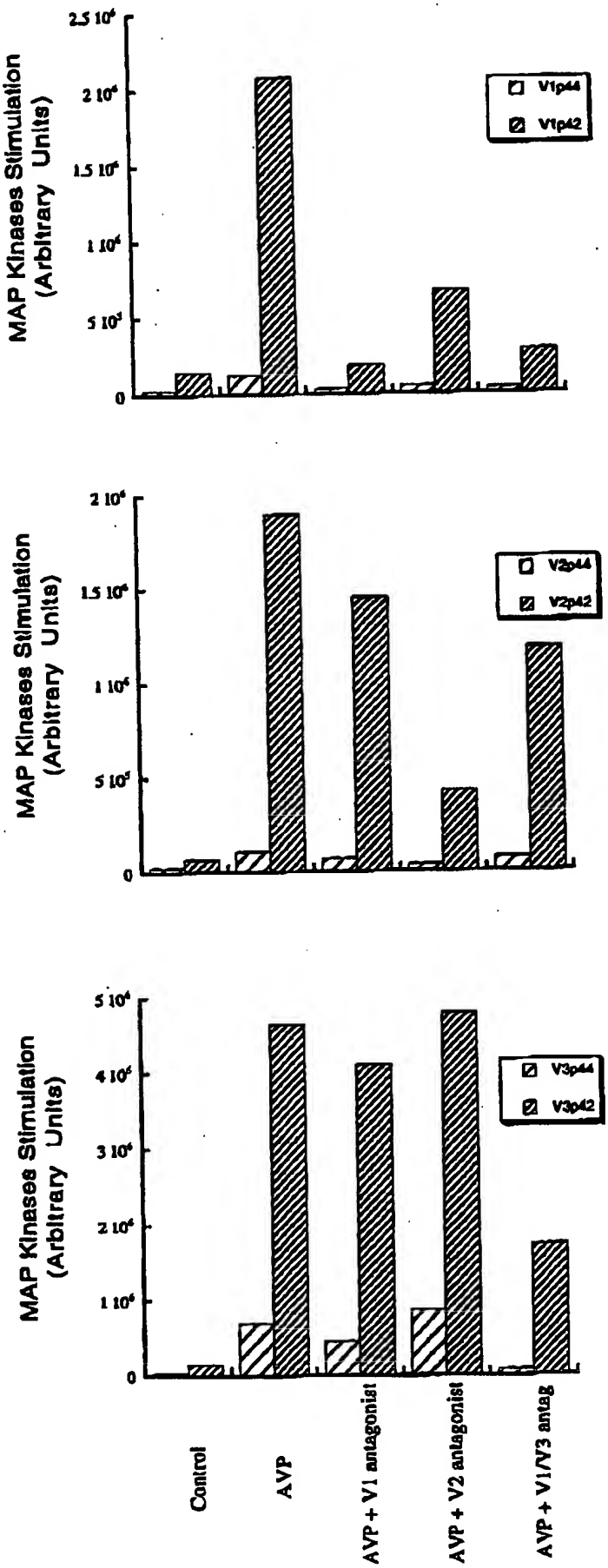


Figure 5. AVP-induced phosphorylation of p42 and p44 MAP kinases in CHO cells stably expressing human V1, V2, or V3 receptors. Transfected CHO cells were grown to sub-confluence in 12-well dishes and incubated in serum-free F12 medium, pH 7.4 for 72 hours. The cells were stimulated for 10 min by AVP alone (0.1 μM) or in the presence of the V_1 antagonist $d(CH_2)_5Tyr(Me)AVP$ or the V_2 antagonist $d(CH_2)_5[D\text{-}Ile^2\text{-}Ile^4\text{-}Ala\text{-}NH_2]AVP$ or the V_1/V_3 antagonist Phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-NH_2 (0.1 μM). Immunoblotting was performed with a phospho-specific p44/42 MAPK (Tyr204) antibody. Both the immunoblots (left panels) and their corresponding densitometries (right panels) are shown.

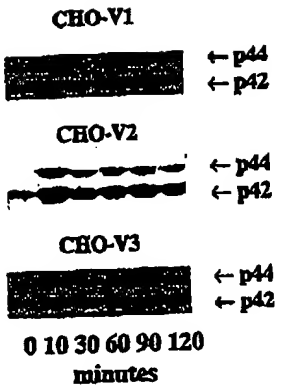


Figure 6. Time course of phosphorylation of p42 and p44 MAP kinases induced by AVP in CHO cells stably expressing human V1, V2, or V3 receptors. Transfected CHO cells were grown to sub-confluence in 12-well dishes and incubated in serum-free F12 medium, pH 7.4 for 72 hours. The cells were stimulated by AVP (10^{-7} M) for varying times and immunoblotting was performed with a phospho-specific p44/42 MAPK (Tyr204) antibody.

growth and differentiation is complex and opposing effects have been proposed (65): activation of MAP kinase by the βγ-subunit of G_s and inhibition of MAP kinase by cAMP-dependent protein kinase activation following G_s-dependent activation of adenylyl cyclase. The V_3Rs presumably activate the MAP kinase pathway through activation of $G_{q/11}$ in a PLC- and PKC-dependent fashion (48). The OTRs have been shown to activate MAP kinase through activation of G_i in a pertussis toxin-sensitive manner (50).

The relationship between MAP kinase activation and cell proliferation was examined by performing experiments with a MEK inhibitor, PD98059. Incubation of CHO-V3 cells with 10 μM PD98059 completely prevented AVP-induced activation of p42/p44, while resulting in only a partial inhibition in AVP-induced [^{3}H]thymidine uptake (Fig. 7 and 8). This suggests that the effect of AVP on cell proliferation implies the recruitment of both p42/p44 MAP kinase-dependent and -independent pathways. This opens the possibility that occupancy of AVP receptors by agonist may trigger simultaneous activation of other kinase cascades, such as p38 or SAPK/JNK, which are more critical for the proliferative response. As a matter of fact, AVP stimulation of V_3Rs of CHO-V3 cells did not activate the p38 MAP kinase pathway (western blotting with a phospho-specific p38 MAP kinase Tyr 182 antibody), but produced an activation of the SAPK/JNK c-jun pathway (western blotting with a phospho-specific SEK1/MKK4 Tyr 705 antibody).

The effect of cyclic AMP on the MAP kinase pathway and cell proliferation depends on both the cell type and the type of tyrosine kinase-receptor involved (66). PKA-dependent inhibition of ras/raf could indeed mediate the attenuation of AVP-induced cell proliferation by forskolin. Thus, we performed experiments measuring the effect of forskolin treatment on AVP-dependent MAPK activation in CHO-V3 cells. As shown on figure 9, forskolin treatment resulted in a partial inhibition of MAPK activation by AVP. This find-

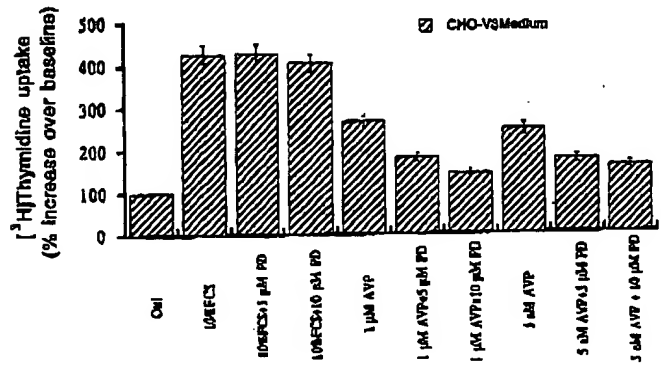


Figure 7. Effects of MEK inhibition on AVP-dependent stimulation of DNA synthesis in CHO-V3 cells. CHO-V3 cells were grown to sub-confluence in 24-well dishes and incubated in serum-free F12 medium, pH 7.4 for 72 hours. The cells were stimulated overnight by 10% FCS or different concentrations of AVP alone or in the presence of the MEK inhibitor PD98059 (5 or 10 μM), followed by addition of [^{3}H]thymidine for 45 minutes. DNA precipitation and radioactivity counting.

Figure 8. Effects of MEK inhibition on AVP-induced phosphorylation of p42 and p44 MAP kinases in CHO-V3 cells. Transfected CHO-V3 cells were grown to sub-confluence in 12-well dishes and incubated in serum-free F12 medium, pH 7.4 for 72 hours. The cells were subsequently incubated for 10 min with 10^{-7} M AVP alone or in the presence of the MEK inhibitor PD98059 (10 μM). Immunoblotting was performed with a phospho-specific p44/42 MAPK (Tyr204) antibody.

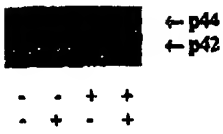


Figure 9. Effects of forskolin treatment on phosphorylation of p42 and p44 MAP kinases induced by AVP in CHO-V3 cells. Transfected CHO cells were grown to sub-confluence in 12-well dishes and incubated in serum-free F12 medium, pH 7.4 for 72 hours. Treatment with forskolin (10 μM) was performed during the last 24 h of serum depletion. The cells were stimulated by 100 nM AVP for 10 min, and immunoblotting was performed with a phospho-specific p44/42 MAPK (Tyr204) antibody.

ing and the preceding paragraph suggest that: a) AVP recruits both MAPK-dependent and -independent pathways leading to cell proliferation, b) elevation of cAMP levels blocks both pathways, c) the MAPK-independent pathway appears quantitatively more important.

Receptor Density-Dependent Signaling Pathways of the Human V3-Pituitary Receptor

Functional characterization of GPCRs (e.g. β1- and β2-adrenergic receptors, luteinizing hormone receptor and the AVP V_2-renal receptor) in mammalian cell lines indicates that a single receptor type can activate multiple second messenger pathways through interaction with one or more G proteins (67–69). In this context, the endogenous human TSH receptor not only activates G_s and $G_{q/11}$, but also members of the G_i and G_{12} families, indicating a complex modulation of downstream pathways (70). This pluripotential appears to be modulated by receptor density (67–69), as shown by the direct correlation between increased expression of G_s-coupled receptors and their ability to stimulate PLC in stably transfected L fibroblasts (68). Thus, GPCRs have the potential to couple to multiple signaling pathways, with the activation of a specific pathway being dependent on both the receptor density and the agonist concentration. These multiple pathways may play an important role in regulating the effectiveness of the signals in different tissues.

The V_3-pituitary receptor is a corticotrophic phenotypic marker which is overexpressed in ACTH-hypersecreting tumors (36, 60). Studies of the agonist/antagonist binding profile and signal transduction pathways linked to the human V_3R have been limited because of the scarcity of this protein. To define the signals activated by V_3Rs and the eventual changes triggered by developmental or pathological receptor regulation, we developed CHO-V_3 cells stably expressing low, medium or high levels of human V_3Rs (B_{max}: <10 pmol/mg, 10 to 25 pmol/mg, and 25 to 100 pmol/mg, respectively).

AVP induced a concentration-dependent increase in the formation of inositol phosphates, thus demonstrating a functional coupling of the V_3R to the phospholipase C pathway. The efficacy of AVP increasing IP_1 production was directly proportional to the level of expression of V_3R in transfected cells (47, 63 and 74% of total labeled phosphoinositides for V3Low, V3Medium and V3High, respectively). EC_{50} values were similar for the various clones (2.85, 3.41, and 1.98 nM for V3Low, V3Medium, and V3High, respec-

tively). Pretreatment of cells with PTX (0.5 μg/ml) resulted in partial inhibition of production of inositol phosphates in response to AVP regardless of V_3R densities (approximately 40% and 15% inhibition for 1 and 100 nM AVP, respectively), suggesting coupling to both G_i and $G_{q/11}$ in this response.

AVP induced an increase in the release of arachidonic acid, indicative of a functional coupling of the V_3R to the phospholipase A_2 pathway. Concentration-response curves for the release of arachidonic acid triggered by AVP showed an approximately four-fold increase in efficacy in the $CHOV_3$Medium compared to the $CHOV_3$Low, together with similar potencies (1.08 and 1.48 nM, respectively). Interestingly, CHO-V3High clones displayed a different response to AVP, characterized by a stimulatory effect peaking at approximately 1 nM and an efficacy lower than that detected in the $CHOV_3$Medium. This suggests that the release of arachidonic acid by the human V_3R is the net result of several factors which come into play in relation to the expression level of V_3Rs. The effect of PTX treatment on the release of arachidonic acid by the human V_3R was dependent on receptor expression (insensitive in CHO-V3Low and partially sensitive in the other two cell types). This could be interpreted as an indication of recruitment of different G proteins and/or forms of PLA_2, depending on the levels of expression of V_3Rs.

Thymidine uptake was measured in CHO cells stably transfected with the AVP V_3R to examine the mitogenic properties resulting from different levels of expression of V_3R. Treatment of both CHO-V3Low or CHO-V3Medium clones with AVP induced a concentration-dependent increase of thymidine uptake, the efficacy of the CHO-V3 Medium clone (E_{max} = +284%, EC_{50} = 2.01 nM) being higher than that of the CHO-V3Low clone (E_{max} = +162%, EC_{50} = 0.66 nM). Interestingly, CHO-V3High clones displayed a biphasic response to AVP characterized by a stimulatory effect in the 0.1–0.5 nM range and an inhibitory effect at higher concentrations. Our previous data suggested that activation of adenylyl cyclase may indeed mediate the inhibitory action of high concentrations of AVP on thymidine uptake in CHO-V3High cells. Hence, we directly measured AVP-stimulated formation of cyclic AMP in CHO cells expressing various levels of V_3Rs. Stimulation of CHO-V3High cells, but not CHO-V3Low or CHO-V3Medium, with 10^{-7} M AVP resulted in a significant increase in cAMP levels (35-fold increase).

We also assessed the potency and efficacy of AVP at activating the MAP kinases as a function of V_3R density in CHO cells stably expressing high, medium or low densities of V_3-pituitary receptors. The phosphorylation of p42 and p44 induced by AVP was concentration-dependent (EC_{50} = 0.48–1.34 nM), and its efficacy directly proportional to the level of expression of V3Rs (Fig. 10). The phosphorylation of p42 and p44 induced by stimulation of V_3Rs with 100 nM AVP was long-lasting and directly proportional to the level of expression of V3Rs. In CHO-V3Low and CHO-V3Medium cells, the effect of AVP peaked at 10 min and started to decay slowly afterwards. In CHO-V3High cells, the effect of AVP peaked at 30–90 min and lasted for at least 5 hours.

The phosphorylation of p42 and p44 induced by stimulation of V_3Rs was not significantly altered by pretreatment with 0.5 μg/ml PTX, thus ruling out G_i involvement (Fig. 11). Conversely, the phospholipase C inhibitor neomycin induced a concentration-dependent reduction of AVP action. The protein kinase C inhibitor staurosporine (0.5 μM) completely abolished the effect of AVP. Down-regulation of protein kinase C following a 24 h exposure to the phorbol ester TPA (1 μM) produced an inhibition of AVP action similar to that observed with staurosporine. A 10-min exposure to TPA produced a marked phosphorylation of p42 and p44 which was prevented by down-regulation or inhibition of protein kinase C, but not by neomycin treatment. The increased phosphorylation triggered by fetal calf serum was not hampered following chronic exposure to TPA.

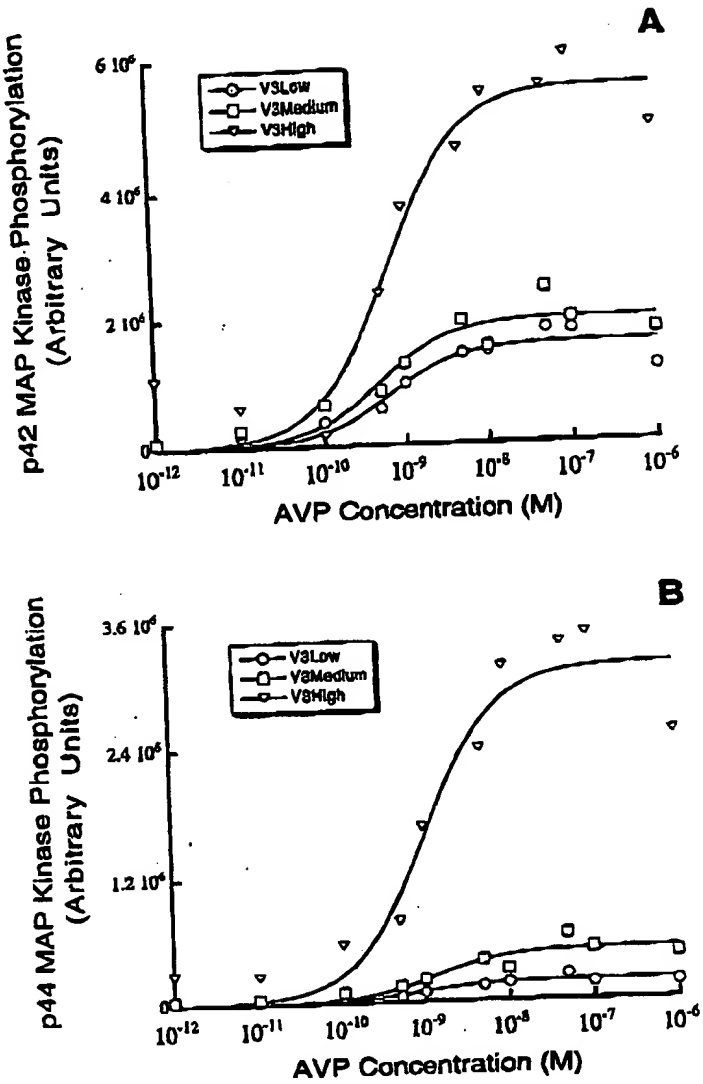


Figure 10. Concentration-response curves corresponding to AVP-induced phosphorylation of p42 and p44 MAP kinases in CHO cells stably expressing high (CHO-V3High), medium (CHO-V3Medium) or low (CHO-V3Low) densities of V_3-pituitary receptors. Transfected CHO cells were grown to sub-confluence in 12-well dishes, followed by incubation in serum-free F12 medium, pH 7.4 for 72 hours. The cells were stimulated for 10 min with AVP (10^{-12} to 10^{-6} M) and immunoblotting was performed with a phospho-specific p44/42 MAPK (Tyr204) antibody. Results show in panels *A* and *B* represent the densitometry of p44 and p42 bands respectively in the immunoblot.

From these experiments in CHO cells expressing various densities of V_3Rs, several conclusions can be drawn. More than one G protein seems to participate in signal transduction pathways linked to V_3Rs. The pattern of activation of a given signal is dependent on both the level of V_3R expression and the concentration of agonist. For some cellular responses including activation of phospholipase C and MAP kinases, the degree of stimulation by AVP is directly proportional to both the concentration of agonist and the level of expression of V_3Rs. Moreover, only G protein(s) of the $G_{q/11}$ class seem to be involved in these signals. For other cellular responses, including activation of phospholipase A_2, cyclic AMP production and thymidine uptake, high levels of V_3R expression seem to couple simultaneously to both stimulatory and inhibitory components in the presence of high concentrations of AVP. Also several G proteins including G_s, G_i and $G_{q/11}$ classes mediate recruitment of these pathways. The increased synthesis of DNA and cAMP levels observed in cells expressing medium and high levels of V_3Rs, respectively, may represent important events in the induction and phenotype maintenance of ACTH secreting tumors.

The currently known intracellular signal pathways linked to activation of the V_1-vascular, V_2-renal, V_3-pituitary, and OT receptors are depicted on figures 12 to 15.

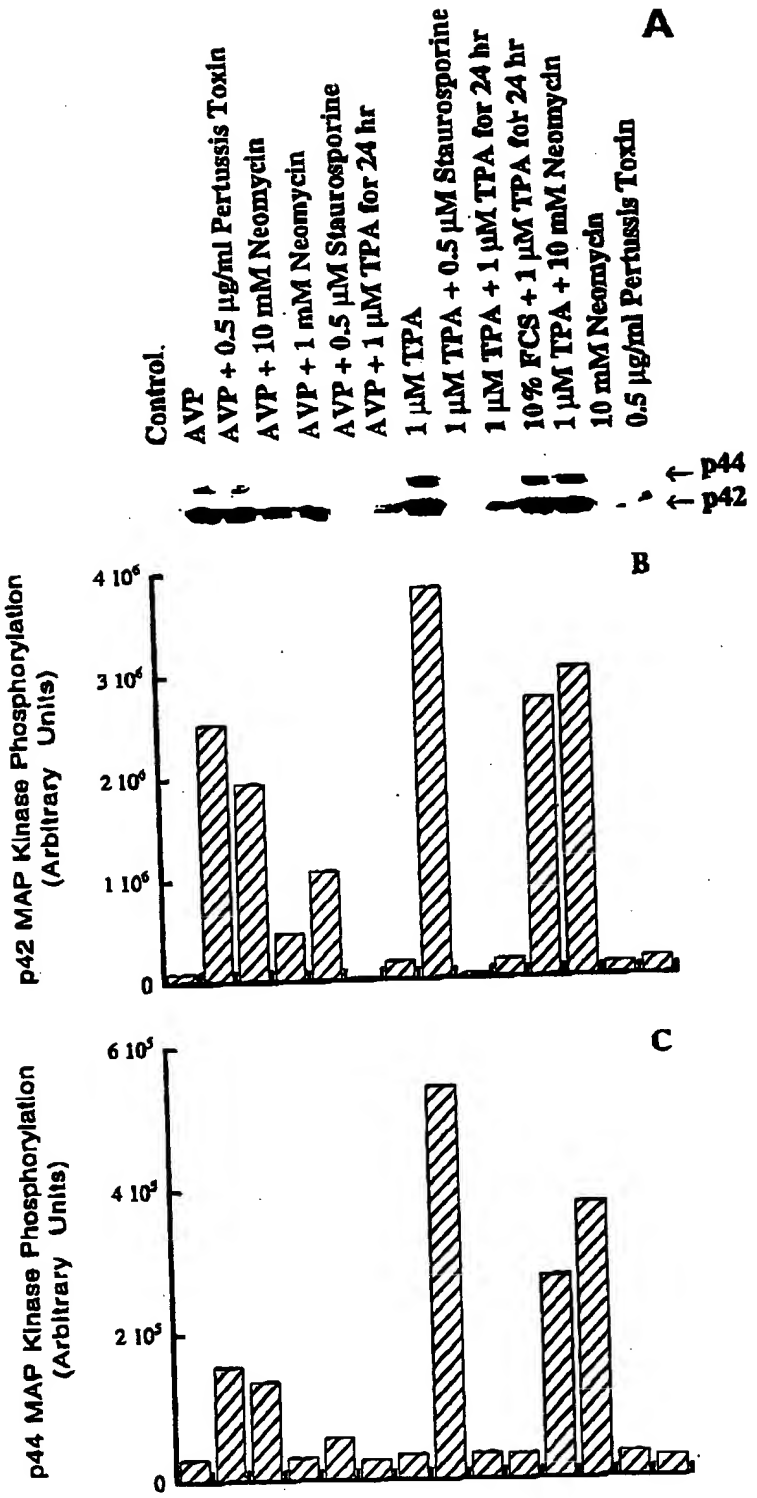


Figure 11. Pathways involved in activation of p42 and p44 MAP kinases by AVP in CHO cells stably expressing AVP V_3-pituitary receptors. Transfected CHO-V3High cells prepared were grown to sub-confluence in 12-well dishes, followed by incubation in serum-free F12 medium, pH 7.4 for 72 hours. The cells were treated with either vehicle, PTX (0.5 µg/ml), or TPA (1 µM) during the last 24 h of culture in serum-free medium. Cells were subsequently stimulated for 10 min with 10^{-8} M AVP in the presence or absence of the indicated compounds. Immunoblotting was performed with a phospho-specific p44/42 MAPK (Tyr204) antibody. Panel A depicts exposure following ECL reaction; panels *B* and *C* represent densitometry of p44 and p42 bands, respectively.

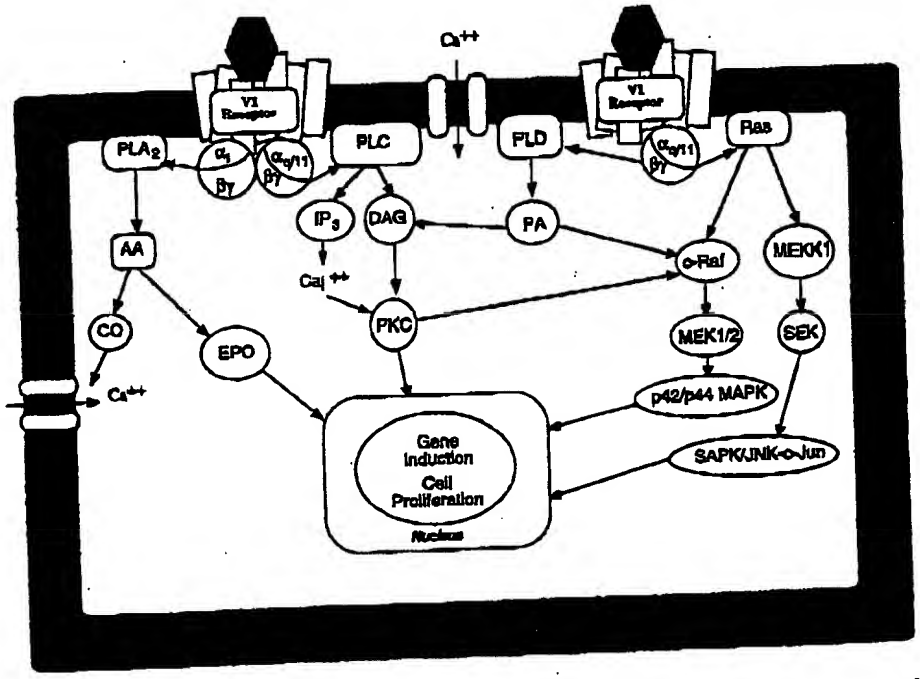


Figure 12. Intracellular pathways coupled to the activation of the human AVP V_1-vascular receptor. Abbreviations are: α, alpha subunits and βγ, beta-gamma dimers of G proteins; PLC, phospholipase C; PLD, phospholipase D; PLA_2, phospholipase A_2; IP_3, inositol-1,4,5 trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; AA, arachidonic acid; CO, cyclooxygenase; EPO, epoxygenase; PA, phosphatidic acid; ERK, extracellular signal-regulated kinase; MEK, mitogen activated ERK kinase; MAPK, mitogen activated protein kinase; SEK, SAPK/ERK kinase; cAMP, cyclic AMP; PKA, protein kinase A.

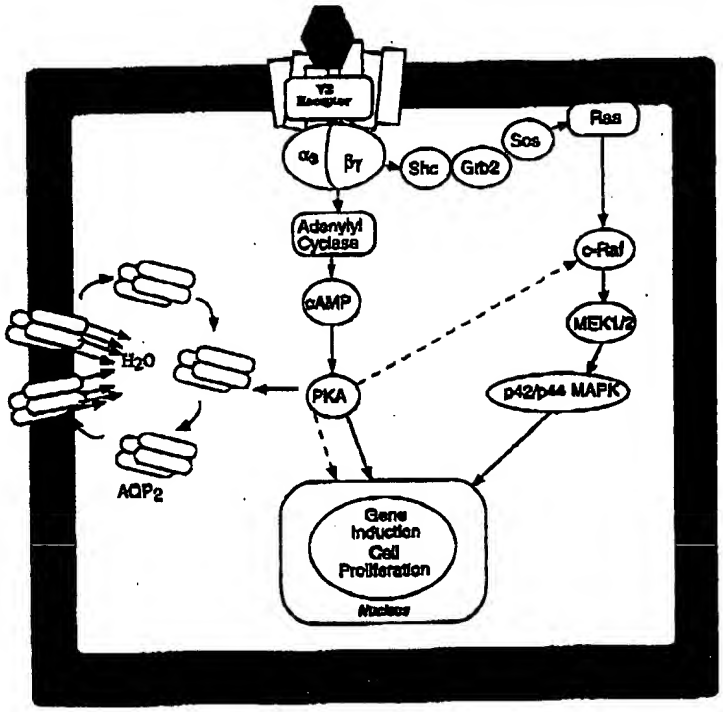


Figure 13. Intracellular pathways coupled to the activation of the human AVP V_2-renal receptor. For abbreviations, see legend of figure 12.

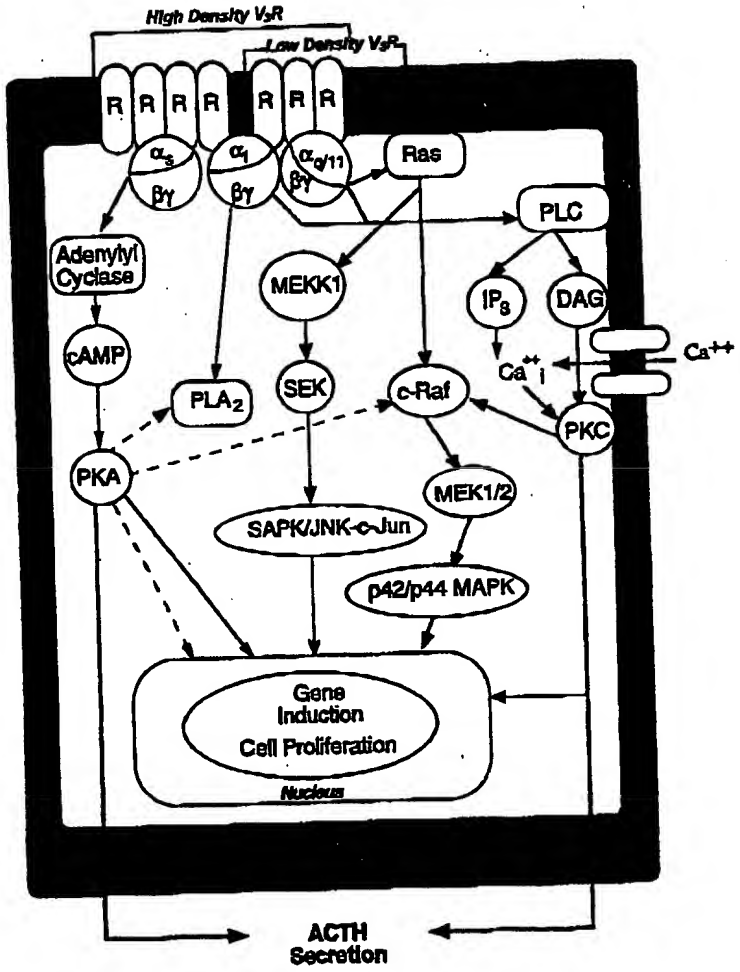


Figure 14. Intracellular pathways coupled to the activation of the human AVP V_3-pituitary receptor. For abbreviations, see legend of figure 12.

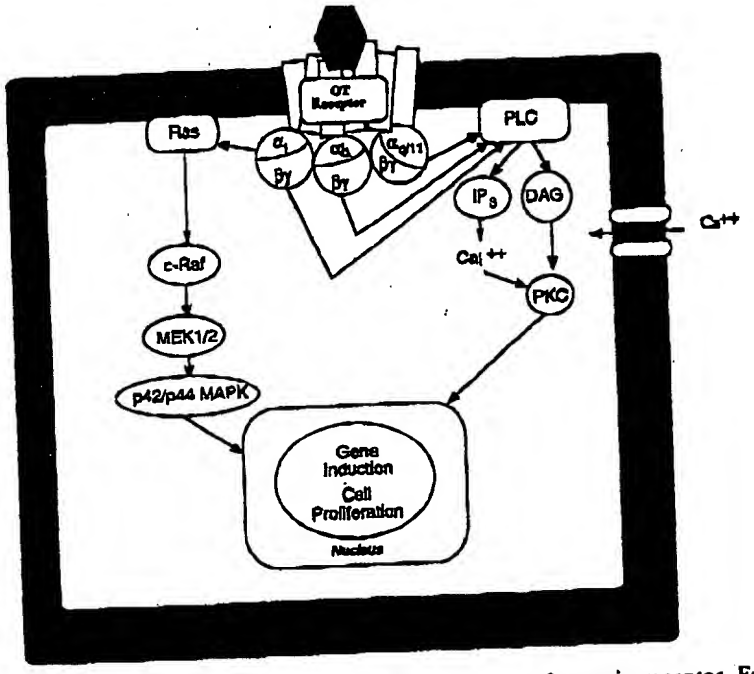


Figure 15. Intracellular pathways coupled to the activation of the human Oxytocin receptor. For abbreviations, see legend of figure 12.

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Serial No.: **09/750,373**
Date Filed: **December 28, 2000**
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